

Dean L. Engelhardt et al.

Serial No.: 08/479,997

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Page 14 [Amendment Under 37 C.F.R. §1.116 to the July 18, 2000  
Office Action - January 18, 2001]

#### REMARKS

Claims 454-575 were previously pending in this application. Claims 454-455, 459, 461, 466, 476, 482-483, 487, 489, 494, 504, 508, 510-531, 533, 535-559, 561 and 563-567 have been amended. Claims 568-575 have been canceled hereinabove. No claims have been added. Accordingly, claims 454-567 as amended are presented for further prosecution on the merits.

A new title of the invention that is believed to be more descriptive of Applicants' claimed invention has been added above. The new title is "Oligo- or Polydeoxyribonucleotides and Oligo- or Polynucleotides Comprising Phosphate Moiety Non-Radioactively Labeled Modified Nucleotides."

In a sincere effort to advance prosecution of this application by reducing or simplifying the issues, Applicants have canceled claims 568-575 above. These claims were the subject of a new matter rejection under 35 U.S.C. §112, first paragraph, which is treated in further detail *infra*.

In a further sincere effort to define their invention more clearly, Applicants have amended several claims above. First, independent claims 454, 482, 511 and 539 have been amended to recite that each such composition is complementary to a nucleic acid of interest or a portion thereof . . . In addition, the polymeric nucleic acid in these independent claims is now defined as comprising at least one modified nucleotide. Further, each of these independent claims has been amended to recite that Sig comprises a non-radioactive label moiety which can be directly or indirectly detected when attached to the phosphate or when the nucleotide is incorporated into the oligo- or polyribonucleotide or when the oligo- or poly(deoxy)nucleotide is hybridized to said complementary nucleic acid of interest or a portion thereof.

In the case of independent claims 511 and 539, the preamble has also been amended to recite "[a]n oligo- or polynucleotide" in place of the former language, "an oligo- or polyribonucleotide." To conform with the aforementioned preamble change in claims 511 and 539, each of the dependent claims (512-531, 535-538, 540-559 and 563-567) has likewise been amended to recite "an oligo- or polynucleotide." In the case of claim 483, the term "polynucleotide" in line 2 has

been changed to "polydeoxyribonucleotide" to conform with the preamble of claim 482 from which it depends. Furthermore, the proviso language in both claims 511 and 539 has been amended to recite that when said oligo- or polynucleotide is an oligoribonucleotide or a polyribonucleotide, and when Sig is attached through a chemical linkage to a terminal PM at the 3' position of a terminal ribonucleotide, said chemical linkage is not obtained through a 2',3' vicinal oxidation of a 3' terminal ribonucleotide previously attached to said oligoribonucleotide or polyribonucleotide. The latter language, particularly the vicinal oxidation recitation, is believed to conform more closely with that in the specification. See the specification, page 53, first full paragraph.<sup>1</sup> The foregoing claim changes, particularly those with respect to the preamble and the language regarding hybridization between the oligo- or poly(deoxy)nucleotide and the complementary nucleic acid of interest follow the discussions held at the August 24, 2000 interview.<sup>2</sup>

Other dependent claims have been amended as follows. Claims 455 and 512 have been amended to recite "wherein said Sig is or renders the nucleotide or the oligo- or polydeoxyribonucleotide" (claim 455) ["or the oligo- or polynucleotide" (in the case of claim 512) "self-signaling or self-indicating or self-detecting." The foregoing changes serve to conform claims 455 and 512 to the language already present in claims 483 and 540, respectively.

A relatively minor symbol change has been made to each of claims 459, 487, 516 and 544. Here, the term " $\alpha$ -position" has been substituted for "alpha-position." A similar symbol change has also been made in the case of claims 461, 489, 518 and 546 where the same term (" $\alpha$ -position") has been substituted, this time, for "delta-position." This latter substitution conforms the claim language with the specification (page 11, second paragraph). Other changes to claims 461, 489,

<sup>1</sup> The first full paragraph on page 53 in the specification discloses:

Broadly, in another aspect of the practices of this invention various methods are useful for the tagging or labeling of DNA in a non-disruptive manner. For example, biotin is added on the end of a DNA or RNA molecule. The addition of biotin is accomplished by addition of a ribonucleotide. The 3',4' vicinal hydroxyl groups are oxidized by periodate oxidation and then reduced by a borohydride in the presence of biotin hydrazide. Alternatively, carbodiimide can also be used to couple biotin to the aldehyde group.

<sup>2</sup> In the August 24, 2000 Interview Summary, the following description was written: Applicant's position is the prior art does not attach labels but merely fragments of proteins and that the resulting product can not be used as a probe.

518 and 544 are discussed *infra*.

In yet a further effort to define their invention more clearly and to reduce the issues related to the new matter rejection (35 U.S.C. §112, first paragraph), Applicants have also amended claims 466, 494, 523 and 551. Previously directed to Sig being complexed with a binding protein therefor and such binding protein being conjugated to ferritin, these claims have now been recast in Markush language. Each claim recites "wherein Sig is selected from the group consisting of a ligand and a specific ligand binding protein." The foregoing language is taken from the specification (pages 101-102) which recites:

The detection of nucleic acids to which specific molecules have been covalently attached can be effected through the use of many naturally occurring proteins to which small molecules are known to specifically bind. In this procedure the small molecules are bound to the nucleotides using the allyl amine side chain. These nucleotides are then incorporated into specific nucleic acids using a DNA or RNA polymerase or ligase chain reaction or a chemical linkage. After annealing this probe with a complementary antiprobe sequence, the presence of the probe is assayed for by the specific binding of the protein to the ligand covalently bound to the probe.

Examples of protein-ligand reactions that are appropriate for this type of detector system include:

...

4. Specific ligand binding proteins included in the transport of small molecules. An example of this is the periplasmic binding proteins of bacteria which have been shown to bind many amino acids, glucose, galactose, ribose and other sugars, Pardee, A. Science, 162:632:637 (1968); G. L. Hazelbaur, and J. Adler, Nature New Bio. 230:101-104 (1971)).

In the above-mentioned examples the ligand bound to the nucleic acid reacts with a naturally occurring protein. The specificity of this reaction resides in the ligand-binding site of the protein.

In addition to the minor word change (" $\alpha$ -position" for " $\delta$ -position") discussed above, a structural formula for one of the chemical linkages recited in claims 461, 489, 518 and 546 has also been corrected, again to conform the language with the specification (page 11, second and third lines from the bottom of the page). In claims 476, 504, 533, 561, the term "anti-Sig immunoglobulin" has been changed to -- anti-hapten immunoglobulin -- . In addition to rendering the claims more definite, the foregoing amendments are believed to eliminate any

possible new matter issues under 35 U.S.C. §112, first paragraph, by conforming the claim language with the specification, to wit, page 33, last paragraph, through page 34, first paragraph:

While a single-step "antibody sandwich" method in which one chromosome spread is challenged, post-hybridization, with rabbit anti-biotin IgG may succeed, this protocol may not generate sufficient fluorescence for unambiguous gene assignments. . . Since one also has available monospecific guinea pig anti-DNP IgG, we can haptenize this secondary antibody with biotin and thus generate two anti-hapten IgG populations, DNP-labeled anti-biotin IgG and biotin-labeled anti-DNP IgG. . . [emphasis added]

Clarifying changes have also been made to five claims (480, 508, 535, 536, 537 and 565). In four of these claims (480, 508, 537 and 565), the word "oxygen" has been substituted for "hydrogen." Thus, claims 480 and 537 now recite "wherein the sugar moiety of said terminal nucleotide has an oxygen atom at each of the 2' and 3' positions thereof." Similarly, claims 508 and 565 now recite "wherein both y and z of said terminal nucleotide comprise an oxygen atom at each of the 3' and 2' positions thereof, respectively." In the case of claim 535, the terms "nucleotide" and "polynucleotide" have been substituted for "ribonucleotide" and "polyribonucleotide," respectively. A similar change has been made to claim 536 where "nucleotide" has been inserted in place of "ribonucleotide." It is believed that the foregoing changes to claims 480, 508, 535, 536, 537 and 565 serve to define more clearly Applicants' claimed invention, particularly with respect to the nature of the labeled terminal nucleotide which can be either a ribonucleotide or a deoxyribonucleotide.

Finally, claims 510 and 567 have been amended both with respect to their chemical structural formula and the definition of the "m" and "n" integers. The structures in claims 510 and 567 now designate the base moiety as "BASE" (and not as "A"). Further, the integers "m" and "n" are now defined as representing "integers from 0 up to about 100,000." The designation of the base moiety as "BASE" merely conforms both dependent claims with the language in claims 482 and 539, from which claims 510 and 567 depend, respectively. Thus, the change is necessary to avoid an issue of improper antecedent basis. The new definition of "m" and "n" is also necessary in order to define both terms in the first instance which are in claims 510 and 567. Thus, a problem of definiteness will be avoided



by allowing this new language which conforms with the specification, pages 23 and 24. See in particular, page 24, lines 4-5 ("wherein m and n represent integers from 0 up to about 100,000").

Entry of the above amendments to the claims is believed to be appropriate and necessary. First, these amendments do not raise new issues which would require further consideration and/or search by the Examiner. In particular, the amendments to the independent claims (454, 482, 511 and 539) with respect to the "probe" aspects of Applicants' claimed nucleic acid compositions follow and incorporate the substance of the August 24, 2000 Interview as set forth in the Interview Summary and discussed in Footnote 2 above. Further, no issue of new matter is raised by the entry of these amendments. Moreover, it is believed that the amendments will actually serve to place this application in better form for appeal by materially reducing or simplifying the issues for appeal. Finally, the amendments do not present additional claims without cancelling a corresponding number of finally rejected claims. In fact, eight claims (569-575) have been canceled above for the purpose of materially reducing or simplifying the issues for appeal. Entry of the amendments is respectfully requested.

Before addressing the substantive issues in the July 18, 2000 Office Action, Applicants would like to acknowledge their appreciation to Examiner Scott Houtteman for the time and courtesy he extended to Drs. Dean L. Engelhardt and Cheryl H. Agris, and to Applicants' undersigned attorney at the interview held on August 24, 2000.

**The Rejection Under 35 U.S.C. §112, First Paragraph**

Claims 459-472 and 474-575 stand rejected for new matter under 35 U.S.C. §112, first paragraph. In the Office Action (page 2), the Examiner stated:

Support was not found were indicated in the specification, nor elsewhere, for the following limitations in Claims 459-472 and 474-575:

Claims 459-463, specific chemical compositions of linkages;  
Claims 464-472, 482-569, specific identity of labels and points of attachment of the "SIG" moiety to internal phosphates;

Claims 474-477 and 570-575, the "composition" limitation, in addition to the above identified limitations.

The new matter is respectfully traversed.

In response, Applicants would like to point out that with respect to claims 568-575, the new matter has been rendered moot in light of the cancellation of these claims. Thus, the remarks below are directed to the new matter rejection insofar as it applies to the remaining pending claims cited in the Office Action.

Ever mindful of the pronouncements on the issue of written description issued in the form of the December 21, 1999 Revised Interim Guidelines (64 FR 71427) and more recently, the January 5, 2001 Guidelines for Examination of Patent Applications Under 35 U.S.C. § 112, ¶ 1, "Written Description" Requirement, Applicants have engaged the services of a patent attorney and former scientist, Dr. Cheryl H. Agris, to address, among others, the new matter rejection. Dr. Agris is submitting a Declaration on behalf of the Applicants.<sup>3</sup> Dr. Agris is a well-respected patent practitioner who has also written and lectured on many patent issues, including written description, enablement and obviousness. Dr. Agris was also a predoctoral fellow who worked in research in the laboratory of Drs. Paul S. Miller and Paul O. P. Ts'o,<sup>4</sup> Department of Biochemistry at the Johns Hopkins University. A copy of Dr. Agris's Declaration is attached to this Amendment as Exhibit A.

After describing her professional work, academic background and research experience in Paragraphs 1-8 (pages 1-4), Dr. Agris points out in Paragraph 9 (pages 4-6) that she has been engaged by the assignee of this application as a scientific and legal consultant to review the prosecution history of U.S. Patent Application Serial No. 08/479,997, filed on June 7, 1995. Dr. Agris also notes that she is being compensated for her review and for making her Declaration. In addition to her review of the prosecution history, Dr. Agris notes that she has also read and reviewed the January 5, 2001 Guidelines for Examination of Patent Applications Under 35 U.S.C. § 112, ¶ 1, "Written Description" Requirement, and

<sup>3</sup> The complete title of Dr. Agris's Declaration is "Declaration of Dr. Cheryl H. Agris, Attorney At Law (In Support Of The Written Description, Enablement & Non-Obviousness Of The Invention Claimed In U.S. Patent Application Serial No. 08/479,997)."

<sup>4</sup> Drs. Miller and Ts'o's 1981 Biochemistry paper ("Biochemical and Biological Effects of Nonionic Nucleic Acid Methylphosphonates," Biochem. 20(7):1874-1880 (1981)) is the subject of an obviousness rejection under § 112, also discussed *infra* and in Dr. Agris's Declaration (Exhibit A).

that her remarks, opinions and conclusions with respect to the written description rejections have been rendered in light of those Guidelines.

After expressing her understanding of the claims to be presented in this application in Paragraph 10 (pages 6-8) of her Declaration (Exhibit A), Dr. Agris reiterates the new matter rejections from the July 18, 2000 and February 3, 1999 Office Actions in Paragraphs 11 and 12 (page 9). In Paragraph 17 (page 12), Dr. Agris defines the level of skill in the art to which the present invention pertains. Based upon her own training, background and experience, Dr. Agris submits that at the time this application was first filed in June 1982, a person of ordinary skill in the art relevant to the subject matter being claimed, including nucleic acid modification, synthesis, hybridization and detection, would have possessed or could have been actively pursuing an advanced degree in organic chemistry and/or biochemistry. Such an ordinarily skilled person could also be at least approaching or ranging toward the level of a junior faculty member with 2-5 years of relevant experience, or at least be a postdoctoral student with several years of experience. Dr. Agris considers herself to possess the level of skill and knowledge of at least a person of ordinary skill in the art to which the present application and invention pertains.

Later, in Paragraphs 18-30 (pages 12-28), Dr. Agris addresses each of the three issues set forth in the written description rejection. For the sake of accuracy and completeness, Dr. Agris's statements are set forth below.

As characterized by Dr. Agris in Paragraph 18 (page 12) of her Declaration (Exhibit A), the written description rejection concerns three issues:

- A. the specific chemical compositions of linkages recited in claims 459-463;
- B. the specific identity of labels and points of attachment of the "SIG" moiety to internal phosphates as recited in claims 464-472 and 482-569; and
- C. the composition limitations as recited in claims 474-477 and 570-575.

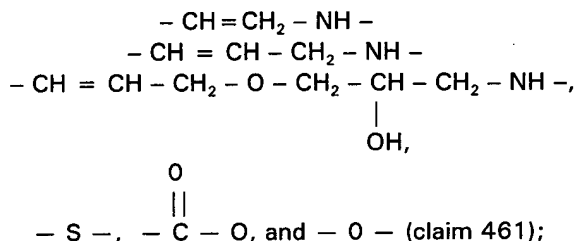
**A. Specific chemical compositions of linkages (claims 459-463)**

In Paragraph 19 (page 13), Dr. Agris indicates her understanding that claims 459-463 are directed to subject matter where

the chemical linkage comprises a member selected from the group consisting of an olefinic bond at the  $\alpha$ -position relative to the point of attachment to the nucleotide, a  $-\text{CH}_2\text{NH}-$  moiety, or both (claim 459);

the chemical linkage comprises an allylamine group (claim 460);

the chemical linkage comprises or includes an olefinic bond at the  $\alpha$ -position relative to the point of attachment to the nucleotide, or any of the moieties:



the chemical linkage of Sig includes a glycosidic linkage moiety (claim 462); and PM is a monophosphate, a diphosphate or a triphosphate and said Sig moiety is covalently attached to said PM through a phosphorus atom or phosphate oxygen (claim 463).

In Paragraph 20 (pages 13-14), Dr. Agris avers that as a person skilled in the art to which the present invention pertains, she have reviewed the '997 specification as originally filed and that it is her opinion and conclusion that the following portions of that disclosure support the above recited chemical linkages:

<u>Chemical Linkage(s)</u>	<u>Citation In Specification</u>	<u>Description in Disclosure</u>
olefinic bond at the $\alpha$ -position relative to the point of attachment to the nucleotide, a $-\text{CH}_2\text{NH}-$ moiety, or both	Page 11, 2nd ¶ Original Claim 78  Page 11, last ¶ Original Claim 79	that the chemical linkage include an olefinic bond at the $\alpha$ -position relative to B  that the chemical linkage group . . . have the structure $-\text{CH}_2\text{NH}-$ , . . .
an allylamine group	Page 11, last ¶ Original Claim 80	Examples of preferred linkages derived from allylamine . . .

<u>Chemical Linkage(s)</u>	<u>Citation In Specification</u>	<u>Description in Disclosure</u>
olefinic bond at the $\alpha$ -position relative to the point of attachment to the nucleotide or any of the moieties $\begin{array}{l} \text{--CH=CH}_2\text{--NH--} \\ \text{--CH=CH--CH}_2\text{--NH--} \\ \text{--CH=CH--CH}_2\text{--O--CH}_2\text{--CH--CH}_2\text{--NH--} \\ \quad \quad \quad   \\ \quad \quad \quad \text{OH} \end{array}$	Page 11, 2nd ¶  Page 11, line 29 Page 11, l. 29-30	preferred that the chemical linkage include an olefinic bond at the $\alpha$ -position relative to B. The presence of such an $\alpha$ -olefinic bond . . .  $\begin{array}{l} \text{--CH=CH--CH}_2\text{--NH--} \\ \text{--CH=CH--CH}_2\text{--O--CH}_2\text{--CH--CH}_2\text{--NH--} \\ \quad \quad \quad   \\ \quad \quad \quad \text{OH} \end{array}$
$\begin{array}{c} \text{--S--} \\ \\ \text{O} \\    \\ \text{--C--O} \\ \\ \text{--O--} \end{array}$	Original Claim 82  <i>ibid.</i>  <i>ibid.</i>	$\begin{array}{c} \text{--S--} \\ \\ \text{O} \\    \\ \text{--C--O} \\ \\ \text{--O--} \end{array}$
glycosidic linkage moiety	Original Claim 25	said Sig chemical moiety is attached by or includes a glycosidic linkage moiety.
PM is a monoP, diP triP and said Sig moiety is covalently attached to said PM through a phosphorus atom or phosphate oxygen	Page 9, lines 8-14  Page 57, Ex. V	wherein each of x, y and z represents $\begin{array}{c} \text{H--}, \text{HO--}, \text{O} \\    \\ \text{HO--P--O--} \dots \\   \\ \text{OH} \end{array}$ Biotin and polybiotinylated poly-L-lysine were coupled to oligoribonucleotides using a carbodiimide coupling procedure described by Halloran and Parker, <u>J. Immunol.</u> , 96 373 (1966).

Dr. Agris concludes Paragraph 20 by indicating that it is her opinion and conclusion as a person skilled in the art that the above-cited portions in the disclosure fully support the various chemical linkages recited in the pending claims of this application. According to Dr. Agris, the above-cited portions describe such recited chemical linkages in sufficient detail that one skilled in the art can reasonably conclude that the inventors had possession of such chemical linkages.

**B. Specific identity of labels and points of attachment of the "SIG" moiety to internal phosphates (claims 464-472 and 482-569)**

In Paragraph 21 (pages 15-16), Dr. Agris expresses her understanding that claims 464-472 and 482-567 (claims 568-569 having been canceled) are directed to subject matter for the following labels of the "SIG" moiety:

**Labels (SIG)**

**Claims 464, 492, 521 and 549** (Listed as (i) through (xvi) below)

- (i) biotin
- (ii) iminobiotin
- (iii) electron dense component  
ferritin (claims 465, 493, 522 and 550)
- (iv) ligand and a specific ligand binding protein (claims 466, 494, 523 and 551 as amended)
- (v) magnetic component  
magnetic oxide (claims 467, 495, 524 and 553)  
ferric oxide (claims 468, 498, 525 and 552)
- (vi) enzyme or an enzyme component  
alkaline phosphatase, acid phosphatase,  $\beta$ -galactosidase, ribonuclease, glucose oxidase and peroxidase (claims 469, 497, 526 and 554);
- (vii) hormone or a hormone component
- (viii) metal-containing component  
catalytic (claims 470, 498, 527 and 555)
- (ix) fluorescent component  
fluorescein, rhodamine and dansyl (claims 471, 499, 528 and 556)
- (x) chemiluminescent component
- (xi) antigen
- (xii) hapten
- (xiii) antibody or an antibody component  
antigen or hapten capable of complexing with an antibody or antibody component specific thereto, and an antibody or antibody component capable of complexing with an antigen or hapten (claims 472, 500, 529 and 557);

- (xiv) composition comprising the oligo- or polydeoxyribonucleotide . . . , a polypeptide capable of forming a complex with Sig and a moiety which can be detected when such complex is formed  
(claims 502, 531 and 559);  
polypeptide comprises polylysine (claims 503, 532 and 560);  
polypeptide is selected from the group consisting of avidin, streptavidin and anti-hapten immunoglobulin (claims 504, 533 and 561);
- (xv) composition of . . . , wherein Sig is a ligand and said polypeptide is an antibody thereto (claims 505, 534 and 562); and
- (xvi) Sig is or renders the nucleotide or the oligo- or polynucleotide self-signaling or self-indicating or self-detecting (claims 512 and 540).

In Paragraph 22 (pages 16-19), Dr. Agris avers that she has reviewed the '997 specification as originally filed and that it is her opinion and conclusion as a skilled person in the art that the following portions of that disclosure support the above recited labels or SIG listed as items (i) through (xvi):<sup>5</sup>

<u>Label (Sig)</u>	<u>Citation In Specification</u>	<u>Description in Disclosure</u>
biotin	Page 10, last 2 lines Page 97, last ¶, thru Page 98, 1st 4 lines Original Claim 92	. . . the preferred A moieties are biotin and iminobiotin. . . . the chemical moiety A . . . is functionally the equivalent of the Sig component or chemical moiety . . . of this invention. . . Sig chemical moiety is biotin
iminobiotin	<i>ibid.</i>	
electron dense component	Page 97, 1st ¶	The Sig moiety might also include an electron dense component, . . .
ferritin	<i>ibid.</i>	such as ferritin, . . .

<sup>5</sup> As noted in footnote 2 (page 16) of Dr. Agris's Declaration, "The citations and descriptions listed below are not necessarily intended to be exhaustive of all the support for any given label or Sig. Rather, the citations and descriptions are offered as illustrative support which is non-limiting."

<u>Label (Sig)</u>	<u>Citation In Specification</u>	<u>Description in Disclosure</u>
ligand and a specific binding protein	Page 101, thru Page 102	. . . presence of probe is assayed for by the specific binding of the protein to the ligand . . .
magnetic component	Page 97, 1st ¶	magnetic component associated or attached thereto, . . .
magnetic oxide	<i>ibid.</i>	such as a magnetic oxide,
ferric oxide	<i>ibid.</i>	or magnetic iron oxide, . . .
enzyme or an enzyme component:	Page 96, 2nd ¶	The Sig moiety could comprise an enzyme or enzymic material
alkaline phosphatase	<i>ibid.</i>	such alkaline phosphatase,
acid phosphatase	Original Claim 41 Original Claim 197	said enzyme is acid phosphatase group . . . acid phosphatase . . .
β-galactosidase	Page 36, 3rd ¶ Also Original Claim 84	direct enzymes such as . . . or β-galactosidase . . . said enzyme is β-galactosidase.
ribonuclease	Page 96, 2nd ¶	or ribonuclease.
glucose oxidase	<i>ibid.</i>	glucose oxidase,
peroxidase	<i>ibid.</i>	horseradish peroxidase,
hormone or a hormone component	Page 102, 1st ¶	3. Hormone receptors and other receptors on the surface of the cell . . .
metal-containing component	Original Claim 28	. . . metal-containing component
catalytic	Original Claim 83 Original Claim 174	said Sig chemical moiety includes or comprises a catalytic metal component . . . catalytically active metal.
fluorescent component	Page 96, 1st ¶	The Sig moiety could include a fluorescing component
fluorescein, rhodamine or dansyl	<i>ibid.</i>	such as fluorescein or rhodamine or dansyl.



<u>Label (Sig)</u>	<u>Citation In Specification</u>	<u>Description in Disclosure</u>
chemiluminescent component	Page 97, 1st ¶	The Sig component or moiety could include . . . a chemiluminescent component
antigen	Original Claim 28 See also Page 88, 2nd ¶	an antigen . . . fix to a solid matrix a specific antigen and bind to this antigen an antibody directed against this antigen which itself has been biotinylated.
hapten	Page 97, 1st ¶	could include a hapten component
antibody or an antibody component	Original Claim 28	or antibody component.
antigen or hapten capable of complexing with antibody . . .	Original Claim 136	said Sig chemical moiety includes an antigenic or hapten component capable of complexing with an antibody specific to said component.
antibody or an antibody component capable of complexing with an antigen or hapten	<i>ibid.</i> See also Page 88, last ¶	The use of the antigen-antibody system for detecting either antigen or antibody is well known.
composition . . . oligo- or polynucleotide . . . polypeptide and moiety which can be detected	Original Claims 167 & 168	A polynucleotide comprising one or more nucleotides . . . coupled to a polypeptide, . . . having attached . . . one or more Sig chemical moieties
polypeptide . . . polylysine	Original Claim 56	said polypeptide is a polylysine.
polypeptide . . . avidin, streptavidin and anti-hapten immunoglobulin	Page 25, last 2 lines Page 26, 3rd ¶  Page 34, 1st ¶	One polypeptide detector for the biotinyl-type probe is avidin. A preferred probe for biotin-containing nucleotides and derivatives is streptavidin, an avidin-like protein . . . We can haptenize this secondary antibody with biotin and thus generate two anti-hapten IgG populations, . . .

<u>Label (Sig)</u>	<u>Citation In Specification</u>	<u>Description in Disclosure</u>
Sig is a ligand and said polypeptide is an antibody	Page 101, 1st ¶	. . . presence of probe is assayed for by the specific binding of the protein to the ligand . . .
self-signaling or self-indicating or self-detecting	Page 82, 1st ¶	Of special importance and significance . . . self-signaling or self-indicating or self-detecting nucleic acids,

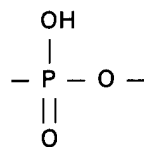
Dr. Agris completes Paragraph 22, indicating that it is her opinion and conclusion as a person skilled in this art that the above-cited portions in the disclosure fully support the various labels which embody Sig in the pending claims of this application. Dr. Agris further indicates that the above-cited portions are in general quite explicit and describe the claimed labels or Sig in sufficient detail that one skilled in the art can reasonably conclude that the inventors had possession of such claimed labels or Sig.

#### Attachment of SIG to Internal Phosphates

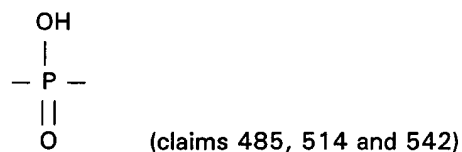
In Paragraph 23 (pages 19-21), Dr. Agris expresses her understanding of the following claims with respect to being directed to the points of attachment of the "SIG" moiety to internal phosphates:

An oligo- or polydeoxyribonucleotide comprising at least one modified nucleotide having the structural formula . . . wherein x, y and z are selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate and wherein Sig is covalently attached directly or through a chemical linkage to at least one phosphate selected from the group consisting of x, y, z, and a combination thereof . . . (claim 482);

oligo- or polydeoxyribonucleotide of . . . wherein said covalent attachment is selected from . . .

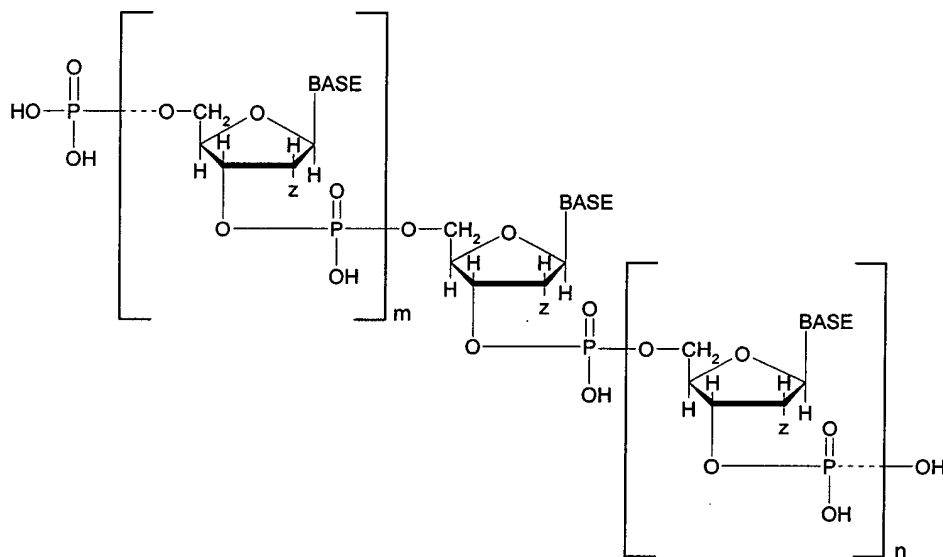


and



oligo- or polydeoxyribonucleotide of . . . wherein said x and y each comprise a member selected from the group consisting of mono-, di or tri-phosphate and said Sig moiety is covalently attached to either or both of said x and y through a phosphorus or phosphate oxygen(claim 491, 520 and 548);

oligo- or polydexoyribonucleotide of . . . having the structural formula:

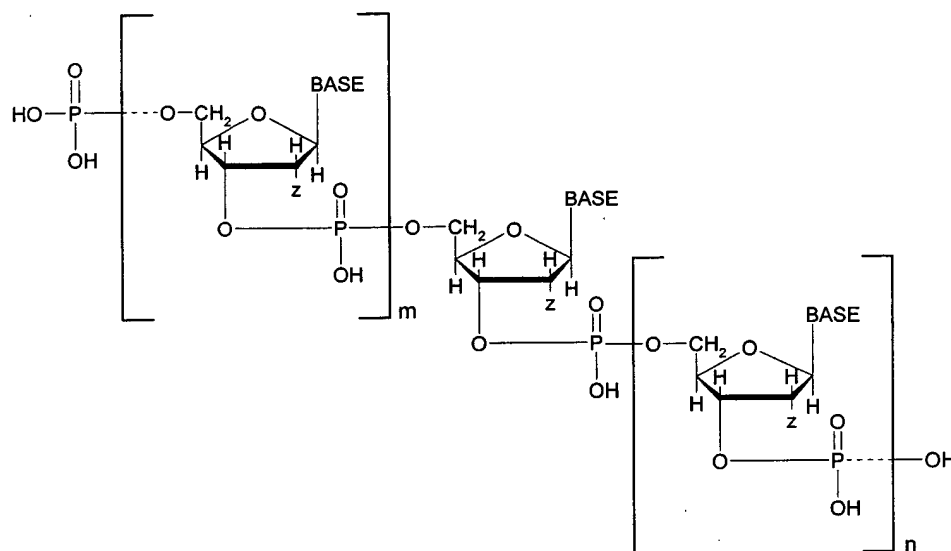


wherein said Sig moiety is attached to at least one of the phosphate moieties in said structural formula (claim 510);

oligo- or polynucleotide . . . having the structural formula Sig - PM - SM - BASE . . . Sig is covalently attached to PM directly or via a chemical linkage (claim 511) [recited in Paragraph 10C above];

oligo- or polyribonucleotide comprising at least one nucleotide having the structural formula . . . Sig is covalently attached to x, y or z directly or through a chemical linkage (claim 539) [recited in Paragraph 10D above]; and

oligo- or polyribonucleotide of . . . , having the structural formula:



wherein said Sig moiety is attached to at least one of the phosphate moieties in said structural formula (claim 567).

In Paragraph 24 (page 21), Dr. Agris states that she has reviewed the '997 specification as originally filed and that as a person of ordinary skill in the art, it is her opinion and conclusion that the original disclosure sufficiently describes the attachment of the Sig moiety to the phosphorus atom as to reasonably convey that Applicants had possession of such claimed subject matter at the time the invention was made in June 1982.

In Paragraph 25 (pages 21-22), Dr. Agris notes as a patent practitioner, she has lectured and written on the requirements of 35 U.S.C. §112, including the Written Description requirements under the first paragraph of §112. Dr. Agris further notes that she has also submitted comments on the Interim Written Description Guidelines issued July 7, 1998, 63 FR 32,639. Dr. Agris points out that Applicants' disclosure and their claimed subject matter also meets the January 5, 2001 Written Description Guidelines (Exhibit 7 of her Declaration), particularly with respect to the attachment of Sig to the phosphorus atom of the phosphate moiety. As set forth by Dr. Agris, those guidelines provide that the written description requirement is met when the patent specification describes the claimed invention in "sufficient detail that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention" (citing *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 19 USPQ2d 1111 (Fed. Cir. 1991) 66 FR 1099 (2001)).

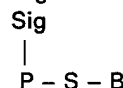
According to these guidelines, possession may be shown by showing that the invention was "ready for patenting" such as by the disclosure of drawings or other descriptions of the invention that are sufficiently specific to enable a person skilled in the art to practice the invention. *Id.* It is well established case law that when the elements recited in the claims are supported by corresponding language in the text, examples, drawings, or other disclosure in the specification, the written description requirement is satisfied and no further analysis is required. *In re Bowen*, 492 F.2d 859 (C.C.P.A. 1974). Furthermore, if new claims are proposed during prosecution, each claim must be expressly, implicitly or inherently supported in the originally filed disclosure and each claim must include all elements which applicant has described as essential. 66 FR at 1105. To establish inherency, it must be clear from any extrinsic evidence provided in the missing descriptive matter is necessarily present in the thing described in the reference and that it would be so recognized by persons of ordinary skill. *In re Robertson*, 169 F.3d 743, 49 USPQ2d 1949 (Fed. Cir. 1999).

Dr. Agris points out in Paragraph 26 (page 22) that training materials have also been provided in connection with the Revised Interim Written Description Guidelines issued on December 21, 1999, and that a decision tree was included with those training materials and is attached to her Declaration as Exhibit 8. According to Dr. Agris, although revisions are expected to the training materials in view of the final Written Description Guidelines, these training materials appear to be still in effect. 66 FR at 1099. When a claim of broader scope is added, the question posed is "Is an element(s) missing from the claim?" If the answer is "yes", the question posed is "Is the missing element(s) described by applicant as being an essential or critical feature of the new claim as a whole?" If the answer is "no", the question posed is "Is there express, inherent or implicit support for the claim as a whole?" If the answer is "yes", the written description requirement is met.

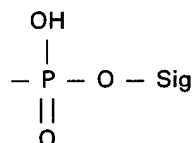
For reasons explained in four subsections (A-D) of Paragraph 27 (pages 22-26), Dr. Agris states that it is her opinion and conclusion that the written description requirement has been met. The four subsections (A-D) of Paragraph 27 are set forth below.

A. First, several structures are depicted and descriptions given where "Sig" is attached to the phosphate moiety. As described on pages 8-10 in the Engelhardt Declaration (Exhibit 6), such structures are found variously in the specification, for example, on page 94, last paragraph, and continuing through page 95, first paragraph:

Still further, nucleotides in accordance with the practices of this invention include the nucleotides having the formula



wherein P is the phosphoric acid moiety, S the sugar moiety and B the base moiety. In these special nucleotides the P moiety is attached to the 3' and/or the 5' position of the S moiety when the nucleotide is deoxyribonucleotide and at the 2', 3' and/or 5' position when the nucleotide is a ribonucleotide. The base B is either a purine or a pyrimidine and the B moiety is attached from the N1 or the N9 position to the 1' position of the sugar moiety when said B moiety is a pyrimidine or a purine, respectively. The Sig chemical moiety is covalently attached to the phosphoric acid P moiety via the chemical linkage



said Sig, when attached to said P moiety being capable of signalling itself or making itself self-detecting or its presence known and desirably the nucleotide is capable of being incorporated into a double-stranded or DNA, RNA or DNA-RNA hybrid.

Later in the specification, on page 96, and continuing through the first paragraph on page 98, other descriptions are provided wherein Sig is attached to the phosphate moiety:

By way of summary, as indicated hereinabove with respect to the make-up of the various special nucleotides in accordance with this invention, the special nucleotides can be described as comprising a phosphoric acid moiety P, a sugar moiety S and a base moiety B, a purine or pyrimidine, which combination of P-S-B is well known with respect to and defines nucleotides, both deoxyribonucleotides and ribonucleotides. The nucleotides are then modified in accordance with the practices of this invention by having covalently attached thereto, to the P moiety and/or the S moiety and/or the B moiety, a chemical moiety Sig. The chemical moiety Sig so attached to the nucleotide P-S-B is capable of rendering or making the resulting nucleotide, now comprising P-S-B with the Sig moiety attached to one or more of the other moieties, self-detecting or signalling itself or capable of making its presence known per se, when incorporated into a polynucleotide, especially a double-stranded polynucleotide, such as double-stranded

DNA, a double-stranded RNA or a double-stranded DNA-RNA hybrid. The Sig moiety desirably should not interfere with the capability of the nucleotide to form a double-stranded polynucleotide containing the special Sig-containing nucleotide in accordance with this invention and, when so incorporated therein, the Sig-containing nucleotide is capable of detection, localization or observation.

The Sig moiety employed in the make-up of the special nucleotides of this invention could comprise an enzyme or enzymic material, such as alkaline phosphatase, glucose oxidase, horseradish peroxidase or ribonuclease. The Sig moiety could also contain a fluorescing component, such as fluorescein or rhodamine or dansyl. If desired, the Sig moiety could include a magnetic component associated or attached thereto, such as a magnetic oxide or magnetic iron oxide, which would make the nucleotide or polynucleotide containing such a magnetic-containing Sig moiety detectable by magnetic means. The Sig moiety might also include an electron dense component, such as ferritin, so as to be available by observation. The Sig moiety could also include a radioactive isotope component, such as radioactive cobalt, making the resulting nucleotide observable by radiation detecting means. The Sig moiety could also include a hapten component or per se be capable of complexing with an antibody specific thereto. Most usefully, the Sig moiety is a polysaccharide or oligosaccharide or monosaccharide, which is capable of complexing with or being attached to a sugar or polysaccharide binding protein, such as a lectin, e.g. Concanavilin A. The Sig component or moiety of the special nucleotides in accordance with this invention could also include a chemiluminescent component.

As indicated in accordance with the practices of this invention, the Sig component could comprise any chemical moiety which is attachable either directly or through a chemical linkage or linker arm to the nucleotide, such as the base B component therein, or the sugar S component therein, or the phosphoric acid P component thereof.

The Sig component of the nucleotides in accordance with this invention and the nucleotides and polynucleotides incorporating the nucleotides of this invention containing the Sig component are equivalent to and useful for the same purposes as the nucleotides described in the above-identified U.S. patent application Serial No. 255,223. More specifically, the chemical moiety A described in U.S. patent application Serial No. 255,223 is functionally the equivalent of the Sig component or chemical moiety of the special nucleotides of this invention. Accordingly, the Sig component or chemical moiety of nucleotides of this invention can be directly covalently attached to the P, S or B moieties or attached thereto via a chemical linkage or linkage arm as described in U.S. patent application Ser. No. 255,223, as indicated by the dotted line connecting B and A of the nucleotides of U.S. Serial No. 255,223. The various linker arms or linkages identified in U.S. Ser. No. 255,223 are applicable to and useful in the preparation of the special nucleotides of this invention.

B. Furthermore, as set forth on pages 10-11 of the Engelhardt

Declaration (Exhibit 6), there are nine separate instances where Sig is described in the specification as being attached to the phosphate moiety P (as well as the sugar moiety S and/or the base moiety B):

<u>Specification</u>	<u>Description</u>
page 90, last paragraph	. . . and a signalling chemical moiety Sig covalently attached thereto, either to the P, S or B moiety.
page 93, first paragraph	. . . include a chemical moiety Sig covalently attached to the P, S and/or B moieties.
page 96, first paragraph	. . . by having covalently attached thereto, to the P moiety and/or the S moiety and/or the B moiety, a chemical moiety Sig.
page 98, first paragraph	. . . the Sig component or chemical moiety of nucleotides of this invention can be directly covalently attached to the P, S or B moieties or attached thereto via a chemical linkage or linkage arm . . .
page 103, first full paragraph	. . . and the signalling or self-detecting moiety, Sig, covalently attached to either the P, S or B moieties, as indicated hereinabove, . . .
page 104, first paragraph	. . . nucleotides in accordance with this invention containing the above-described components P, S, B and Sig . . .
page 105, first paragraph	. . . the nucleotides of this invention include the P, S, B and Sig components wherein the Sig is covalently attached to either the P, S or B moieties
page 105, second paragraph	The moiety Sig attached to the special nucleotides of this invention containing the other moieties or components P, S, B provide a site per se for the attachment thereto, the Sig component, . . .
page 106, first paragraph	. . . the special P, S, B and Sig-containing nucleotides of this invention, . . .

Upon seeing such structures and their descriptions in the specification, one of ordinary skill in the art would clearly understand that "Sig" may be attached to either the oxygen or phosphorus atom of the phosphate moiety. As described in the Engelhardt Declaration (Exhibit 6), methods are provided in the specification and are well known in the art for attaching a substituent to the phosphorus and to the oxygen atom. Therefore, in [Dr. Agris's] opinion, the written description



requirement has been met.

C. Thirdly, [Dr. Agris holds] the same opinion and [she has] reached the same conclusion in applying the analysis set forth in the decision tree in the training materials (Exhibit 8) provided in connection with the Written Description Guidelines. As a person of ordinary skill in the art, [Dr. Agris] also [recognizes] that the scope of claims 459-472 and 474-567 is somewhat broader than the original claims. Specifically, the originally filed claims (see, for example, original claims 141) contained the requirement that "Sig" be attached to the oxygen atom in the phosphate moiety (PM). The currently pending claims do not contain such a requirement. This missing element is not an essential or critical feature of the new claims as a whole because there is no specific requirement set forth in the specification that "Sig" be attached to the oxygen atom in the phosphate moiety.

D. Further, in [Dr. Agris's] opinion and conclusion, there is inherent support for the subject matter of claims 459-474 and 474-567. As a person of ordinary skill in the art, it is [Dr. Agris's] opinion and conclusion that a reading of the specification, including Example V and the extrinsic evidence detailed in the Engelhardt Declaration (Exhibit 6), reasonably conveys that in accordance with the present invention, when a substituent is depicted as being attached to a phosphate moiety (PM), that such a substituent could be attached to either an oxygen or phosphorus atom.

At the end of Paragraph 27 (page 26), Dr. Agris states that it is her opinion and conclusion that Applicants' claimed subject matter wherein the Sig moiety is attached to the phosphorus or oxygen atom of the phosphate moiety meets the requirements for written description.

**C. Composition limitations (claims 474-477 and 570-575)**

In Paragraph 28 (pages 26-27), Dr. Agris directs her remarks to claims 474-477 which contain the following subject matter:

a composition comprising the oligo- or polydeoxyribonucleotide of claim 454, a polypeptide capable of forming a complex with Sig and a moiety which can be

detected when such complex is formed (claim 474);

the composition . . . wherein said polypeptide comprises polylysine (claim 475);

the composition . . . wherein said polypeptide is selected from the group consisting of avidin, streptavidin and anti-Sig immunoglobulin (claim 476); and

the composition . . . wherein Sig is a ligand and said polypeptide is an antibody thereto (claim 477).

In Paragraph 29 (page 27), Dr. Agris states that she has reviewed the '997 specification as originally filed and that it is her opinion and conclusion as a skilled person in the art that the following portions of that disclosure support the above recited compositions:

<u>Composition(s)</u>	<u>Citation In Specification</u>	<u>Description in Disclosure</u>
comprising oligo- or polydeoxyribonucleotide . . . , a polypeptide capable of forming a complex with Sig and a moiety which can be detected when such complex is formed	Page 8, last ¶  Page 97, last ¶, thru Page 98, 1st 4 lines	wherein A represents a moiety . . . which is capable of forming a detectable complex with a polypeptide . . . the chemical moiety A described in U.S. patent application Serial No. 255,223 is functionally the equivalent of the Sig component or chemical moiety . . . of this invention.
wherein said polypeptide comprises polylysine	Original Claim 56	said polypeptide is a polylysine.
polypeptide . . . avidin, streptavidin and anti-hapten immunoglobulin	Page 25, last 2 lines Page 26, 3rd ¶  Page 34, 1st ¶	One polypeptide detector for the biotinyl-type probe is avidin. A preferred probe for biotin-containing nucleotides and derivatives is streptavidin, an avidin-like protein . . . We can haptenize this secondary antibody with biotin and thus generate two anti-hapten IgG populations, . . .
Sig is a ligand and said polypeptide is an antibody	Page 101, 1st ¶	. . . presence of probe is assayed for by the specific binding of the protein to the ligand . . .

In Paragraph 30 (page 27, through the first line on page 28), Dr. Agris states

that based upon the above-cited portions, it is her opinion and conclusion as a person skilled in the art that the specification as originally filed fully supports the compositional subject matter in claims 474-477. Dr. Agris further states that she finds that the disclosure provides sufficient detail that a person skilled in the art can reasonably conclude that the inventors had possession of the subject matter in claims 474-477 at the time the application was originally filed in June 1982.

In view of the submission of Dr. Agris's Declaration (Exhibit A), Applicants respectfully request reconsideration and withdrawal of the new matter rejection.

#### The Rejection Under 35 U.S.C. §112, First Paragraph

Claims 454-575 stand rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention, for reasons of record. In the Office Action (pages 3-4), the Examiner stated:

Halloran et al., J. Immunol. 96(3):373-378, 1966 (Halloran) discloses the attachment of a specific signal moiety, a protein, to the phosphorus atom of the phosphate moiety using a specific linker, a -C-(CH<sub>2</sub>)<sub>4</sub>-N- chain. In contrast, the claims are drawn to a much broader category, a generic "SIG" moiety and linkage, and specific compounds such as those recited in claim 464-magnetic, hormone, metal containing "SIG" moieties, for example.) Thus, the scope of the enablement is not commensurate with the scope of the claims.

In addition, several of the reference articles are drawn to labeling a mononucleotide and express doubt about labeling an oligonucleotide. Armstrong et al., Eur. J. Biochem. 0:33-38, 1976, teaches that the labeled mononucleotides are "strong competitive inhibitors" of the reaction which is necessary to produce a labeled oligonucleotide from a labeled mononucleotide. See Armstrong, p. 33, col. 1. This reaction is the use of the labeled mononucleotide as a substrate for the polymerase mediated synthesis of the oligonucleotide.

While Armstrong teaches that some labeled mononucleotide will be incorporated, Armstrong teaches no guidance as to which of the myriad labels within the scope of these claims will function in the claimed invention. Lacking any guidance in the specification and in view of the breadth of the claims, it would require undue experimentation in order to enable a reasonable number of embodiments of these claims. The skilled artisan would have to

experiment with various SIG moieties, linkages and attachment sites, as well as various reaction protocols and protecting groups.

The enablement rejection is respectfully traversed.

In response, Applicants offer the Declaration of Dr. Cheryl H. Agris (Exhibit A) and her statements on the enablement issue.

In Paragraph 31 (page 28) of her Declaration (Exhibit A), Dr. Agris states that as a person of at least ordinary skill in the art to which the present invention pertains, it is her opinion and conclusion that the original disclosure of the '997 specification was enabling and permitted the practice of the subject matter of claims 454-567 without undue experimentation. Her reasons for concluding that the '997 specification is enabling are set forth on pages 28 and 29 of her Declaration.

According to Dr. Agris in Paragraph 32 (page 28), although Halloran et al. only teaches one moiety, other moieties are disclosed in the specification and are known in the art. Specifically, Example V of the instant specification discloses a method for attaching biotin, one of the embodiments for Sig, to the phosphate moiety of a mononucleotide and an oligonucleotide that are coupled to a protein, poly-L-lysine. Furthermore, as detailed on pages 11 and 12 in the Engelhardt Declaration (Exhibit 6), the chemistry and reactions for attaching substituents to the oxygen or phosphorus atoms in a nucleotidyl phosphate or phosphoric acid moiety were already known in the art at the time the initial application was filed in June 1982.

In Paragraph 33 (pages 28-29), Dr. Agris responds to statements made in the July 18, 2000 Office Action regarding the Armstrong et al. reference where it is stated on page 3 in that Office Action that:

Armstrong et al., Eur. J. Biochem. 70:33-38, 1976, teaches that the labeled mononucleotides are "strong competitive inhibitors" of the reaction which is necessary to produce a labeled oligonucleotide from a labeled mononucleotide. See Armstrong, p. 33, col. 1. This reaction is the use of the labeled mononucleotide as a substrate for the polymerase mediated synthesis of the oligonucleotide.

The conclusion is reached in the Office Action that:

. . . Lacking any guidance in the specification and in view of the breadth of the claims, it would require undue experimentation in order to enable a reasonable number of embodiments of these claims. The skilled artisan would have to experiment with various SIG moieties, linkages and attachment sites, as well as various reaction protocols and protecting groups.

Dr. Agris responds to the above in her Declaration, indicating that it is her opinion and conclusion as a person skilled in the art that the labeled mononucleotides referenced in Armstrong et al. were strong competitive inhibitors because unmodified nucleoside triphosphates (NTPs) were present in the assay mixture. This would not be the case or a factor in the present invention according to Dr. Agris. Armstrong et al. actually show that it is possible to incorporate such modified mononucleotides and that modified NTPs may be used as substrates. Although Armstrong et al. does disclose that some modified nucleotides are better than others in terms of binding to RNA polymerase, a person skilled in the art would expect that some routine testing or refinement is necessary.

In light of Dr. Agris's Declaration and her statements on enablement offered as a person skilled in the art, Applicants respectfully request that the enablement rejection be reconsidered and withdrawn.

#### **The First Rejection Under 35 U.S.C. §103**

Claims 454-575 stand rejected under 35 U.S.C. §103 for being unpatentable over Gohlke et al., U.S. Patent No. 4,378,458, filed 3/1981 in view of Sodja et al., Nucleic Acids Research 5(2):385-401 (1978) and further in view of applicant's admissions for reasons of record.

In response to the first obviousness rejection, Applicants offer the Declaration of Ann Sodja, Ph.D.,<sup>6</sup> the co-author of the above cited Sodja et al. 1978 article, and presently a tenured Associate Professor in the Department of Biological Sciences at Wayne State University in Detroit, Michigan.

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<sup>6</sup> The complete title of Dr. Sodja's Declaration is "Declaration of Dr. Ann Sodja (In Support Of The Non-Obviousness Of The Invention Claimed In U.S. Patent Application Serial No. 08/479,997)."

After describing her professional work, academic background, research experience and scientific publications in Paragraphs 1-4 (pages 1-3), Dr. Sodja points out in Paragraph 5 (page 3) that she has been engaged as a scientific consultant by the assignee in order to review the prosecution of this application. Dr. Sodja also states that she is being compensated for the review and the Declaration she is making in this application.

In Paragraph 6 (pages 4-5), Dr. Sodja describes her understanding of the obviousness rejection at hand. In Paragraph 7 (pages 5-8), Dr. Sodja sets forth her understanding of the claimed invention as represented by the independent embodiments (claims 454, 482, 511 and 539). Dr. Sodja defines in Paragraph 9 (page 8) the level of skill in the art. According to Dr. Sodja, based upon her own training, background and experience, she submits that at the time this application was first filed in June 1982, a person of ordinary skill in the art relevant to the subject matter being claimed, including nucleic acid modification, synthesis, hybridization and detection, would have possessed or could have been actively pursuing an advanced degree in organic chemistry and/or biochemistry. Such an ordinarily skilled person could also be at least approaching or ranging toward the level of a junior faculty member with 2-5 years of relevant experience, or would at least be a postdoctoral student with several years of experience. Dr. Sodja considers herself to possess the level of skill and knowledge of at least a person of ordinary skill in the art to which the present application and invention pertains.

In Paragraph 10 (page 8), Dr. Sodja states that it is her opinion and conclusion that the subject matter of claims 454-567 would not have been rendered obvious at the time the invention was made from a combined reading of the Gohlke's '458 Patent in view of her own publication, Sodja et al., and further in view of applicant's admissions for reasons of record. Dr. Sodja's provides her reasons in Paragraphs 11-16 (pages 8-11) of her Declaration.

As the author of the cited Sodja and Davidson (1978) (Exhibit 2 in her Declaration), Dr. Sodja points out in Paragraph 11 (pages 8-9) that her work was intended to help Dr. Davidson and her with electron microscopic gene mapping and gene enrichment of DNA:RNA hybrids. By coupling cytochrome-c to the oxidized 2', 3' terminus of RNA and attaching biotin labels to the coupled cytochrome-c,

Drs. Sodja and Davidson found that electron microscopic gene mapping could be carried out efficiently with avidin-ferritin and avidin-polymethacrylate sphere labels. For their gene mapping studies, Drs. Sodja and Davidson used DNA and RNA from *E. coli* and *Drosophila melanogaster*. Examples of their results obtained with this method are shown by the electron micrographs in Figures 2 and 4 which are published in the cited 1978 paper (Exhibit 2 of Dr. Sodja's Declaration) on pages 393 and 396, respectively. Furthermore, the authors found that gene enrichment was also efficiently obtained by buoyant banding of DNA:RNA-biotin:avidin-spheres in cesium chloride (CsCl) gradient. Results of the authors' enrichment experiments for 5S rRNA from *Drosophila* DNA are presented in Table II on page 398 in Dr. Sodja's 1978 paper (Exhibit 2 of her Declaration).

In Paragraph 12 (page 9), Dr. Sodja points out that at the time when she was conducting experiments related to her 1978 paper, she was neither thinking nor intending to attach a detectable non-radioactive label to the terminus of RNA for the purpose of making a nucleic acid hybridization probe. Rather, after hybridizing the modified RNA with DNA, Dr. Sodja was using large marker molecules, such as avidin-ferritin and avidin spheres, to produce more efficient gene mapping by electron microscopy and gene enrichment by cesium chloride gradient. In her work, Dr. Sodja oxidized the free 2', 3' OH groups of RNA to the dialdehyde form using periodate as described in her 1978 paper both in the reaction scheme outlined on page 386 (no. 1) and in the MATERIALS AND METHODS Section on page 387 under Preparation and Purification of RNA-Cytochrome-c:

tRNA or 5S RNA were heated at 80° for 1-8 min in 1 mM NaAc buffer at pH 6.8, cooled, adjusted to 0.1 M NaAc buffer (pH 4.8) and treated with periodate as previously described (1). The amount of RNA used was 0.5 - 1 mg in 0.5 - 1 ml of reaction mixture.

Dr. Sodja notes that the publication cited as (1) above is Broker et al., "Electron microscopic visualization of tRNA genes with ferritin-avidin:biotin labels," also in Nucleic Acids Research, 5(2):363-384 (1978). A copy of Broker et al. is attached to Dr. Sodja's Declaration as Exhibit 7.

As a person of ordinary skill in the art, Dr. Sodja points out in Paragraph 13 (pages 9-10) that the periodate oxidation method used in her 1978 paper (Exhibit 2 of her Declaration) and in Broker et al. (Exhibit 7 of her Declaration) is applicable

only to RNA which has two vicinal OH groups at the 3' and 2' positions. Other nucleic acids, including DNA, do not possess an OH group on the 2' position. Thus, according to Dr. Sodja, the periodate oxidation method used in her 1978 paper (Exhibit 2) or Broker et al. (Exhibit 7) could not be used to attach a detectable non-radioactive label to DNA as set forth, for example, in claims 454 and 482 (see amendments to independent claims (Exhibit 4) and composite set of claims (Exhibit 5) in this application.

As a person of ordinary skill in the art, Dr. Sodja states in Paragraph 14 (page 10) that it is her opinion and conclusion that the claims 511 and 539 in this application, which claims are drawn to an oligo- or polynucleotide, are outside of her 1978 paper (Exhibit 2) or Broker et al. (Exhibit 7). Dr. Sodja states that as set forth in Paragraph 7C and 7D above, the amendments to the independent claims (Exhibit 4) and the composite set of claims, claims 511 and 539 contain the proviso that

. . . provided that when said oligo- or polynucleotide is an oligoribonucleotide or a polyribonucleotide, and when Sig is attached through a chemical linkage to a terminal PM at the 3' position of a terminal ribonucleotide, said chemical linkage is not obtained through a 2',3' vicinal oxidation of a 3' terminal ribonucleotide previously attached to said oligoribonucleotide or polyribonucleotide.

Clearly, according to Dr. Sodja, the fact that claims 511 and 539 eschew any and all chemical linkages which are obtained through a 2',3' vicinal oxidation of a terminal ribonucleotide, is significant because her 1978 paper relied exclusively on the vicinal oxidation of RNA using periodate. It is Dr. Sodja's opinion and conclusion that the subject matter of claims 511 and 539 would not have been taught or suggested to one of ordinary skill in the art at the time this application was first filed in June 1982 from reading her 1978 paper (Exhibit 2), taken with Gohlke's '458 Patent (Exhibit 6) and any of Applicants' admissions of record. As previously stated, the chemistry disclosed in Dr. Sodja's 1978 paper relied exclusively on vicinal oxidation of the free 2, 3' OH groups of RNA. The subject matter set forth in claims 511 and 539 clearly avoids such chemistry.

In Paragraph 15 (page 10), Dr. Sodja explains that at the time that she conducted the experiments disclosed in her 1978 paper, she was concerned that the chemistry would not work with oligoribonucleotides (10 ribonucleotides or less)



or very short polyribonucleotides. With such short pieces of RNA, she felt at the time that the addition of a large linker, such as cytochrome c, and a large biotin marker, might be too large in comparison to the length of the RNA such that steric hindrance would reduce, if not stymie hybridization between complementary RNA and DNA strands altogether.

Finally, in Paragraph 16 (page 11), Dr. Sodja states her understanding that in the obviousness rejections made in both the February 3, 1999 and July 18, 2000 Office Actions, the Gohlke '458 Patent was cited as the primary reference, and that her 1978 paper was cited as the secondary reference. This was explained on page 5 in the February 3, 1999 Office Action:

it is Gohlke in view of Sodja which is the basis of the rejection. There is no evidence that Gohlke cannot be applied to Sodja for the expected benefit of generating other types of labeled oligonucleotides using the Gohlke labels.

It is Dr. Sodja's opinion and conclusion that even applying Gohlke's disclosed labels to her 1978 paper, one of ordinary skill in the art would not have arrived at the claimed invention in this application, as set forth in claims 454-567. As stated earlier, the chemistry used in Dr. Sodja's 1978 paper could not be applied to nucleic acids, such as DNA that lacked the 2' OH group otherwise found in RNA. Moreover, the claims drawn to the use of a terminal ribonucleotide as a modified nucleotide specifically avoid the vicinal oxidation and periodate chemistry described in Dr. Sodja's 1978 paper. Thus, according to Dr. Sodja, using Gohlke's labels with the chemistry from her 1978 paper, one of ordinary skill in the art would not have arrived at the invention now claimed in this application. Nor, according to Dr. Sodja, would such a person have had a reasonable expectation of success in reaching the present invention from a combined reading of Gohlke's 458 Patent, her 1978 paper and any statements made by the Applicants which are of record in this application.

In view of the submission of Dr. Sodja's Declaration (Exhibit B), Applicants respectfully request reconsideration and withdrawal of the first obviousness rejection.

**The Second Rejection Under 35 U.S.C. §103**

Claims 454-575 stand rejected under 35 U.S.C. §103 for being unpatentable over Halloran et al., J. Immunol. 96(3):373-378, 1966 or Miller et al. 20(7):1874-1880, 1981 for reasons of record. In the Office Action (pages 4-5), the Examiner stated:

Both Halloran and Miller teach specific labels, (SIG moieties such as proteins and thiophosphates) attached to nucleic acids. See Halloran p. 373, Fig. 1 and col. 2; Miller p. 1874, col. 1. These prior art references differ from the claims in the recitation of some specific labels and linkages. It would have been prima facie obvious, however, to one of ordinary skill in the art at the time the invention was made to substitute any linker or label in the methods of Halloran and Miller for the expected benefit of constructing multiple labels. Given the fact that diverse labels such as proteins and thiophosphates, the ordinary artisan would have reasonably expected any moiety used as a label to function in the claimed invention.

Repeating an important point from a previous Office action, applicants arguments to the 35 U.S.C. §112, first paragraph rejections have provided strong evidence of obviousness and vice-versa. For example, the references used to support enablement, Halloran and Miller add evidence of obviousness.

It is important that the arguments for patentability explain, that the prior art supplied by applicant, for example Halloran and Miller, can buttress the specification--providing needed evidence that the thin "SIG Phosphate" disclosure both "describes" and "enables" the detailed invention now claimed--but that these same prior art references do not render the claims obvious.

Furthermore, the criticisms of the obviousness rejections must be made without undermining the enablement rejection. For example, if arguing that Halloran and Miller are somehow "non-enabled" one must justify how the specification can be enabled. After all, the prior art contains much more detail than that found in the specification.

Finally, the specification is held to a higher standard than the teachings of the prior art supplied in an obviousness rejection. As stated in a previous office action:

35 U.S.C. § 112 provides that, in return for the grant of monopoly, the specification must enable one skilled in the art to "make and use" the invention without "undue experimentation" whereas 35 U.S.C. § 103 makes no such requirement. Thus, a teaching of how to use a compound can be entirely adequate to render a claim obvious but, at the same time, entirely inadequate to support the allowance of such a claim.

In response, Applicants again offer the Declaration of Dr. Cheryl H. Agris, Attorney At Law (Exhibit A)

In Paragraph 34 (page 29) of her Declaration (Exhibit A), Dr. Agris states that as a person of at least ordinary skill in the art to which the present invention pertains, it is her opinion and conclusion that the claimed subject matter at hand in the form of claims 454-567 would not have been obvious at the time the invention was made from a reading of either Halloran et al. or Miller et al. Dr. Agris's reasons for the non-obviousness of the invention of claims 454-567 are set forth in Paragraphs 35-39 (pages 29-31) as described below.

Dr. Agris notes in Paragraph 35 (page 29) that it was asserted in the last Office Actions that both Halloran et al. and Miller et al. teach specific labels and that it would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to substitute any linker or label in the methods of Halloran and Miller for the expected benefit of constructing multiple labels. Dr. Agris also notes that in paragraph 11 of the February 3, 1999 Office Action, it was requested that arguments for patentability explain that the prior art supplied by applicant can buttress the specification providing needed evidence that the "SIG-phosphate" disclosure both "describes" and "enables" the detailed invention now claimed, but that these same prior art references do not render the claims obvious.

In Paragraph 36 (29-30), Dr. Agris responds to those statements in the Office Action. As one skilled in the art, it is Dr. Agris's opinion and conclusion that neither Halloran et al. nor Miller et al. actually disclose or suggest a "Sig-phosphate" moiety as defined in the Serial No. 08/479,997 specification. According to Dr. Agris, Halloran et al. is directed to covalently conjugating polynucleotides to proteins as a means of stimulating antibody formation and Miller et al. is directed to non-ionic oligonucleotide analogs. As explained by Dr. Agris, such analogs may be more easily taken up by cells than oligodeoxyribonucleotides and are resistant to cleavage by a variety of nucleases. According to Dr. Agris, neither of these modified polynucleotides were ever made for labeling purposes, nor were such use of Halloran's or Miller's modified polynucleotides ever suggested by the cited documents. Dr. Agris explains that neither the protein in Halloran et al. nor the methyl group in Miller et al. would be considered a detectable label as defined in the art or a detectable Sig chemical moiety as defined in the '997 specification. Before the advent of the invention now claimed, explains Dr. Agris,

there had been no suggestion regarding attaching a Sig group, that is, a moiety capable of non-radioactive detection when attached to the phosphate moiety of at least one nucleotide in an oligo- or polydeoxyribonucleotide or polynucleotide.

As one skilled in the art to which this invention pertains, Dr. Agris points out in Paragraph 37 (page 30) that there had been teachings regarding modifying the phosphate moiety with various substituents, but none of these substituents comprised a Sig-phosphate as set forth in the present claims. Once such a concept for modifying the phosphate moiety of a nucleotide was formulated, according to Dr. Agris, one of ordinary skill in the art would have looked to prior art references in order to develop methods for obtaining the oligo- or poly(deoxyribo)nucleotides of the present invention. As a specific example, Dr. Agris notes that Halloran et al. disclosed methods for coupling proteins to nucleotides and oligonucleotides. This reference may be used, according to Dr. Agris, as a general reference for coupling amino acid moieties to the phosphate moiety. As explained by Dr. Agris, there is no disclosure in Halloran et al., however, that a final oligo- or poly(deoxyribo)nucleotide comprising at least one modified nucleotide of the formula set forth in the pending claims could be obtained.

In Paragraph 38 (pages 30-31) of her Declaration, Dr. Agris states that with respect to the cited Miller reference, she is very familiar with the chemistry employed to make oligonucleoside methylphosphonates, having been a graduate student in Dr. Paul Miller's laboratory during the years 1979-1986. During that time according to Dr. Agris, she and other members of Dr. Miller's group looked to procedures published on obtaining oligonucleotides in order to synthesize oligonucleoside methylphosphonates. For example, the condensing agent used in the cited Miller et al. reference, mesitylene sulfonyl tetrazolide, was used in synthesizing oligonucleotides. A copy of Stawinski et al. [Nucleic Acids Research 4:353-371 (1977)] which describe the use of mesitylene sulfonyl tetrazolide in synthesizing oligonucleotides is attached as to Dr. Agris's Declaration as Exhibit 9. Also attached to Dr. Agris's Declaration is section II.A. of my Ph.D. thesis (Exhibit 2) detailing strategies used in synthesizing oligonucleoside methylphosphonates. Although her thesis was not submitted until January 1986, Dr. Agris notes that the synthetic work described on pages 13-16 actually took place between 1979-1983. Dr. Agris was personally involved in synthesizing oligonucleoside

methylphosphonates in June 1982. As described in those pages from Dr. Agris's thesis, methods known in the art for synthesizing oligodeoxyribonucleotides were used as a basis for synthesizing oligonucleoside methylphosphonates. However, according to Dr. Agris, neither she nor in my opinion, others of ordinary skill in the art had a reasonable expectation of success that these procedures could be successfully used in preparing oligonucleoside methyl phosphonates. For example, Stawinski et al. (Exhibit 9) merely provided a starting point regarding possible reaction conditions that could be used. There is no suggestion in Stawinski et al., however, that arylsulfonyltetrazoles could or even should be used to synthesize oligonucleoside methylphosphonates. Dr. Agris avers that the references cited were primarily used as guidelines for trying to formulate methods for synthesizing oligonucleoside methylphosphonates.

In the final paragraph of her Declaration (39), Dr. Agris states that it appears that the Miller and Halloran references were used as guidelines in formulating the oligo- or poly(deoxyribo)nucleotides of the present invention. The disclosures in each of these references would be sufficiently enabling for one of ordinary skill in the art for formulating procedures for synthesizing the oligo- or poly(deoxy)ribonucleotides of the present invention. According to Dr. Agris, there was no suggestion, however, that these procedures could or should be used to obtain, nor would one of ordinary skill in the art have a reasonable expectation of success in obtaining the oligo- or poly(deoxy)ribonucleotides of the present invention. Thus, it is Dr. Agris's opinion and conclusion that a person of ordinary skill in the art would not have arrived at the invention claimed in U.S. Patent Serial No. 08/479,995 from a reading of the cited Miller or Halloran references, or even by combining the two references.

In light of Dr. Agris's Declaration (Exhibit A) and her statements on the non-obviousness of the present invention, Applicants respectfully request that the second obviousness rejection be withdrawn upon further reconsideration.

\* \* \* \* \*

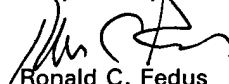
**SUMMARY AND CONCLUSIONS**

Claims 454-566 are presented for further examination. Claims 454-455, 459, 461, 466, 476, 482-483, 487, 489, 494, 504, 508, 510-531, 533, 535-559, 561 and 563-567 have been amended. Claims 568-575 have been canceled hereinabove. Thus, 454-567 as amended are presented for further examination in this application.

This Amendment is accompanied by a Request For An Extension Of Time (3 Months) and authorization for the fee therefor. Also accompanying this Amendment is a Notice of Appeal and authorization for the fee therefor. No other fee or fees are believed due in connection with this filing. In the event that any other fee or fees are due, however, authorization is hereby given to charge the amount of any such fee(s) to Deposit Account No. 05-1135, or to credit any overpayment thereto.

If a telephone conversation would further the prosecution of the present application, Applicants' undersigned attorney request that he be contacted at the number provided below.

Respectfully submitted,



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Registration No. 32,567  
Attorney for Applicants

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# 37

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicant(s):	Engelhardt et al.	)	
Serial No.:	08/479,997	)	Group Art Unit: 1634
Filed:	June 7, 1995	)	Examiner: Scott W. Houtteman
For:	OLIGO- OR POLYNUCLEOTIDES, AND OTHER COMPOSITIONS COMPRISING PHOSPHATE MOIETY LABELED NUCLEOTIDES	)	

Pelham Manor, NY 10803

Honorable Commissioner of Patents and Trademarks  
Washington, D.C. 20231

**DECLARATION OF DR. CHERYL H. AGRIS, ATTORNEY AT LAW  
(IN SUPPORT OF THE WRITTEN DESCRIPTION, ENABLEMENT & NON-  
OBVIOUSNESS OF THE INVENTION CLAIMED IN U.S. PATENT APPLICATION  
SERIAL NO. 08/479,997)**

I, Cheryl H. Agris, hereby declare as follows:

1. I am a solo practitioner in patent law and intellectual property licensing matters, having been so engaged since 1998. Previous to that from 1992 to 1998, I was a patent attorney at Novo Nordisk of North America in New York City. Prior to my position at Novo Nordisk, I was a law clerk in the Biotechnology Group at the law firm of Pennie & Edmonds, also in New York City. I became a patent agent in 1990. As an attorney registered to practice before the U.S. Patent and Trademark Office, my present work involves the preparation and prosecution of U.S. patent applications in the biotechnology, pharmaceutical and chemical fields. I also oversee the foreign prosecution of patent applications. My present work also involves performing patentability and validity studies, infringement analysis and freedom of operation studies. As an intellectual property attorney, I have prepared licensing, consulting and confidentiality agreements for clients. I have also engaged in the peer review of patent prosecution by third parties. My legal and work experience at Novo Nordisk and Pennie & Edmonds is described in my curriculum vitae (CV) which is attached to my Declaration as Exhibit 1.

Dean Engelhardt et al.

Serial No. 08/479,997

Filed: June 7, 1995

Page 2 [Declaration of Cheryl H. Agris, Ph.D., Attorney At Law (In Support of the Written Description, Enablement & Nonobviousness of the Invention Claimed in U.S. Patent Application Serial No. 08/479,997)]

2. Before entering the intellectual property field, I was a scientist and researcher from 1979 - 1988. In 1979, I was an undergraduate research fellow at the Argonne National Laboratory in Argonne, Illinois. There, I analyzed bile acids isolated from the bile, urine, or serum from children with cholestatic liver disease using gas chromatography and gas chromatography/mass spectroscopy. Later from 1979 to 1986, I was a predoctoral fellow (graduate student) in the Division of Biophysics, Department of Biochemistry at the Johns Hopkins University (JHU) in Baltimore, Maryland. My thesis advisors were Dr. Paul O. P. Ts'o, Chair, Division of Biophysics and Dr. Paul S. Miller. While conducting my thesis research in Dr. Paul Miller's laboratory, I helped formulate methods for synthesizing nonionic oligonucleotide analogs and oligonucleoside methylphosphonates. As a member of Dr. Miller's group, I also studied the effects of oligonucleoside methylphosphonate sequences on the synthesis of VSV (vesicular stomatitis virus) proteins in cell culture and in vitro. In connection with my doctorate that I earned at JHU in 1986, I wrote a dissertation titled "Effects of Chemically Synthesized Oligodeoxyribonucleoside Methylphosphonates on Vesicular Stomatitis Virus Protein Synthesis and Infection," a copy of a portion of which is attached as Exhibit 2. During my education and training at JHU, I co-authored a dozen papers with Dr. Miller, these papers being listed on my CV (Exhibit 1). While working in Dr. Miller's laboratory for several years, I became very familiar with the synthesis of oligonucleotides, particularly the nonionic oligonucleotide analogs, oligonucleoside methylphosphonates, including the chemistry disclosed in Dr. Miller's 1981 Biochemistry paper [Biochemistry 20(7):1874-1880 (1981)] cited in the last two Office Actions issued in the above-identified patent application, and discussed *infra*. During the years 1986 - 1988, I was a research fellow at the Sloan Kettering Institute in New York City working in Dr. Robert Krug's laboratory. Dr. Krug was a member at that time of the Molecular Biology Program at Sloan Kettering; his research focused on transcriptional and translational control of influenza viral protein synthesis. While there, I investigated the mechanism of the block in the splicing of influenza viral NS1 mRNA to NS2 mRNA in vitro using molecular biological and biochemical approaches.

3. As indicated in my CV (Exhibit 1), my formal education includes three degree programs. In 1979, I received my Bachelor of Arts in chemistry from Goucher



College in Towson, Maryland. In 1986, I received my doctoral degree (Ph.D.) from the Johns Hopkins University, School of Hygiene and Public Health (Department of Biochemistry, Division of Biophysics). In 1992, I received my *Juris Doctor* degree from the Brooklyn Law School in Brooklyn, New York.

4. Among my honors, awards and fellowships as listed on page 2 of my CV (Exhibit 1), I was a Richardson Scholar at Brooklyn Law School from 1988-1992. I was an American Cancer Society Fellow at the Sloan Kettering Institute between 1986-1988. In 1984, I received a Student Research Award from the Delta Omega Honorary Public Health Society. Between 1979 and 1986, I was the recipient of predoctoral training grants, first as an NIH predoctoral trainee (1979-1982) and later as a predoctoral trainee under the Albert Szent-Gyorgyi Foundation (1982-1986). When I graduated from college in May 1979, I received General Honors and Honors in Chemistry, in addition to having received the Louise Kelly Award in Chemistry. Previous to that, I worked in the Undergraduate Research Program (January - May 1979) and in the Summer Graduate Student Program (June - August 1979) at Argonne National Laboratory.

5. My experience in continuing legal education has covered a number of significant areas in patent law. At various patent meetings and conferences, I have made several oral presentations on patent issues, including, among others, issues related to the written description and enablement requirements under 35 U.S.C. §112, first paragraph. These presentations included the following: "Why Deposit Biological Materials?" New York, New Jersey, Connecticut, and Pennsylvania Joint Seminar on Developments in Patent Law, April 2000; "Inventorship" National Association of Patent Practitioners meeting, July 1999; "What to Claim in Biotechnology Patent Applications" National Association of Patent Practitioners meeting, October 1997; and "*In re Deuel*, Obviousness Standard for Biotechnology" BIO '96, June 1996.

6. Related to my efforts in the field of patent law are a number of faculty appointments and several oral presentations. These include: speaker on Intellectual Property Considerations in "Angel Financing: Navigating the Legal & Business Issues" at the Citybar Center for Continuing Legal Education (CLE), The Association of the Bar of the City of New York, November 28, 2000; organizer and instructor

at the National Association of Patent Practitioner's 2000 Short Course on Nuts and Bolts of Patent Prosecution (July 2000); instructor at the Sixth, Eighth, Ninth and Tenth Annual Patent Prosecution Workshops: Advanced Claim Drafting and Amendment Writing (1996, 1998, 1999, 2000); and speaker at the Law Seminars: Biotechnology Key Legal & Business Issues, November 18-19, 1999 in Seattle, Washington. Each of these faculty appointments are listed on the third page of my CV (Exhibit 1). Among my oral presentations are two held last year and one each in 1999, 1998, 1997 and 1996. These presentations are also listed on page 3 of my CV (Exhibit 1).

7. I have also attended the following continuing legal education programs in the intellectual property area. These include in the year 2000, Writing and Using Intellectual Property Opinions (Association of the Bar of the City of New York), and the International Intellectual Property Symposium at the Brooklyn Law School. In 1999, I attended the legal program Preparing Legal Opinions 1999: Intellectual Property Due Diligence in Business Transactions, also with the Association of the Bar of the City of New York. In 1998, I took part in the New York, New Jersey, Connecticut and Pennsylvania Joint Seminar on Developments in Patent Law. In 1996, I attended "The Basics of Licensing and Licensing Law." I also attended two Patent Resources Group courses, "Advanced PCT Practice" and "European Patent Office Practice." In 1993, I attended the Practising Law Institute (PLI) program on Technology Licensing and Litigation. These are listed on the first page of my CV (Exhibit 1).

8. Among my publications are seven legal-related articles and fourteen scientific papers, including the dozen papers with Dr. Paul Miller referenced above in Paragraph 2. All of these publications are listed on pages 3-5 of my CV (Exhibit 1). Also listed are some representative U.S. patents among the approximately 150 U.S. patents in which I have participated in the preparation and/or prosecution. These representative U.S. patents are listed on page 4 of my CV (Exhibit 1).

9. I have been engaged by Enzo Biochem, Inc. as a scientific and legal consultant in order to review portions of the current prosecution of U.S. Patent Application Serial No. 08/479,997 (presently titled "Oligo- or Polynucleotides, and Other Compositions Comprising Phosphate Moiety Labeled Nucleotides") that was

Dean Engelhardt et al.

Serial No. 08/479,997

Filed: June 7, 1995

Page 5 [Declaration of Cheryl H. Agris, Ph.D., Attorney At Law (In Support of the Written Description, Enablement & Nonobviousness of the Invention Claimed in U.S. Patent Application Serial No. 08/479,997)]

filed on June 7, 1995. I am being compensated by Enzo for this review and for making this Declaration. Included for my review were significant portions of the file wrapper for this application, including the original specification (hereinafter "the '997 specification"), the previously pending claims in this application (454-575), changes to independent claims (454, 482, 511 and 539)<sup>1</sup> to be submitted in a response (Amendment Under 37 C.F.R. §1.116) to the July 18, 2000 Office Action, and the latest composite set of claims (454-567) which will be pending in this application following the submission of the aforementioned Amendment After Final. A copy of the previously pending claims (454-575), the changes to the independent claims (454, 482, 511 and 539), and the latest composite set of claims (454-567) are attached to this Declaration as Exhibits 3, 4 and 5, respectively. I have also reviewed the July 18, 2000 Office Action as well as five other previous Office Actions issued on June 20, 1996, May 13, 1997, January 6, 1998, September 29, 1998 and February 3, 1999. I have also reviewed several papers filed in response to the aforementioned office actions. These papers include Applicants' June 23, 2000 Communication, their June 22, 2000 Second Supplemental Amendment, their June 20, 2000 Supplemental Amendment, their January 4, 2000 Amendment Under 37 C.F.R. §1.115, their January 19, 1999 Supplemental Response, their November 20, 1998 Amendment Under 37 C.F.R. §1.116, their July 6, 1998 Amendment Under 37 C.F.R. §1.115, their November 24, 1997 Amendment Under 37 C.F.R. §1.116, and their December 20, 1996 Amendment In Response To June 20, 1996 Office Action And Request For A Three Month Extension Of Time. I generally agree with the substance of Applicants' remarks and positions as set forth in these aforementioned responses. In particular, I agree with the Declaration of Dr. Dean L. Engelhardt In Support Of Adequate Description and Enablement (hereinafter "the Engelhardt Declaration") that was submitted as Exhibit A to Applicants' November 27, 1997 Amendment Under 37 C.F.R. §1.116 In Response To June 25, 1997 Office Action. A copy of the Engelhardt Declaration is attached to my Declaration as Exhibit 6. I have also reviewed the Examiner Interview Summary Records dated November 3, 1998 and August 24, 2000. I also attended the latter August 24, 2000 interview on behalf of Enzo. Moreover, I have read and reviewed the Guidelines for Examination of

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<sup>1</sup> I understand that several dependent claims have also been amended. The affected dependent claims include 455, 459, 461, 466, 476, 480, 483, 487, 489, 494, 504, 508, 510, 512-531, 533, 535-559, 561 and 563-567. I have also reviewed the amendments to the dependent claims which will also be submitted in Applicants' January 18, 2001 Amendment Under 37 C.F.R. §1.116.

Dean Engelhardt et al.

Serial No. 08/479,997

Filed: June 7, 1995

Page 6 [Declaration of Cheryl H. Agris, Ph.D., Attorney At Law (In Support of the Written Description, Enablement & Nonobviousness of the Invention Claimed in U.S. Patent Application Serial No. 08/479,997)]

Patent Applications Under the 35 U.S.C. 112, ¶1, "Written Description" Requirement, which were published on January 5, 2001 in the Federal Register, Vol. 66, No. 4, Pages 1099-1111. My remarks below, opinions and conclusions with respect to the written description rejections are rendered in light of the aforementioned January 5, 2001 Guidelines. A copy of the January 5, 2001 Guidelines is attached to my Declaration as Exhibit 7.

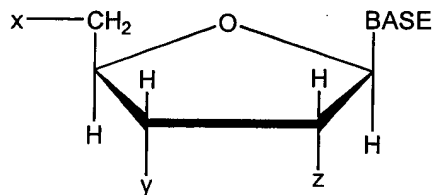
10. As I understand it, the claimed invention as represented by the amended independent claims to be submitted (Exhibit 4) is directed to detectable nucleic acid compositions comprising at least one modified nucleotide.

A. As I understand it, one significant embodiment as set forth in amended claim 454 is an oligo- or polydeoxynucleotide which is complementary to a nucleic acid of interest or a portion thereof. The oligo- or polydeoxynucleotide comprises at least one modified nucleotide having the formula

Sig—PM—SM—BASE

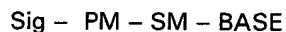
wherein PM is a phosphate moiety, SM is a sugar moiety and BASE is a base moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof. The PM is attached to SM, the BASE is attached to SM, and Sig is covalently attached to PM directly or through a chemical linkage. The element Sig comprises a non-radioactive label moiety which can be directly or indirectly detected when attached to PM or when the modified nucleotide is incorporated into the oligo- or polydeoxynucleotide or when the oligo- or polydeoxynucleotide is hybridized to the complementary nucleic acid of interest or a portion thereof.

B. As set forth in amended claim 482 and as I understand it, another significant embodiment of the claimed invention is an oligo- or polydeoxyribonucleotide which is complementary to a nucleic acid of interest or a portion thereof. The oligo- or polydeoxyribonucleotide comprises at least one modified nucleotide having the structural formula:



In the structural formula, BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, and is attached to the 1' position of the pentose ring from the N1 position when BASE is a pyrimidine or from the N9 position when BASE is a purine or a deazapurine. The substituents x, y and z are selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate. Sig is covalently attached directly or through a chemical linkage to at least one phosphate selected from the group consisting of x, y, z, and a combination thereof. Sig comprises a non-radioactive label moiety which can be directly or indirectly detected when so attached to the phosphate or when the modified nucleotide is incorporated into the oligo- or polydeoxynucleotide or when the oligo- or polydeoxynucleotide is hybridized to the complementary nucleic acid of interest or a portion thereof.

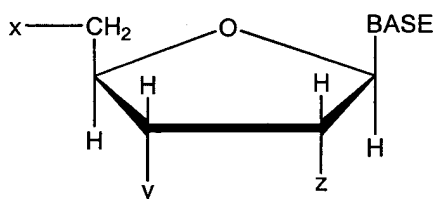
C. I understand that the claimed invention as set forth in amended claim 511 is directed to an oligo- or polynucleotide which is complementary to a nucleic acid of interest or a portion thereof. The oligo- or polynucleotide comprises at least one modified nucleotide having the formula



wherein PM is a phosphate moiety, SM is a sugar moiety and BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof. PM is attached to SM, BASE is attached to SM, and Sig is covalently attached to PM directly or via a chemical linkage. Sig comprises a non-radioactive label moiety which can be directly or indirectly detected when attached to PM or when the modified nucleotide is incorporated into the oligo- or polynucleotide, or when the oligo- or polynucleotide is hybridized to the complementary nucleic acid of interest or a portion thereof. When the oligo- or polynucleotide is an oligoribonucleotide

or a polyribonucleotide, and when Sig is attached through a chemical linkage to a terminal PM at the 3' position of a terminal ribonucleotide, the chemical linkage in this oligo- or polynucleotide is not obtained through a 2',3' vicinal oxidation of a 3' terminal ribonucleotide previously attached to the oligoribonucleotide or polyribonucleotide.

D. I understand another embodiment as set forth in amended claim 539 is an oligo- or polynucleotide which is complementary to a nucleic acid of interest or a portion thereof. The oligo- or polynucleotide comprises at least one modified nucleotide having the structural formula:



wherein BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, and wherein BASE is attached to the 1' position of the pentose ring from the N1 position when BASE is a pyrimidine or from the N9 position when BASE is a purine or a deazapurine. The elements x, y and z are selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate. Sig is covalently attached directly or through a chemical linkage to at least one phosphate selected from the group consisting of x, y and z, and a combination thereof. Sig comprises a non-radioactive label moiety which can be directly or indirectly detected when so attached to the phosphate or when the modified nucleotide is incorporated into the oligo- or polynucleotide, or when the oligo- or polynucleotide is hybridized to the complementary nucleic acid of interest or a portion thereof. Furthermore, when the oligo- or polynucleotide is an oligoribonucleotide or a polyribonucleotide and when Sig is attached through a chemical linkage to a terminal PM at the 3' position of a terminal ribonucleotide, the chemical linkage is not obtained through a 2',3' vicinal oxidation of a 3' terminal ribonucleotide previously attached to the oligoribonucleotide or polyribonucleotide.

Dean Engelhardt et al.

Serial No. 08/479,997

Filed: June 7, 1995

Page 9 [Declaration of Cheryl H. Agris, Ph.D., Attorney At Law (In Support of the Written Description, Enablement & Nonobviousness of the Invention Claimed in U.S. Patent Application Serial No. 08/479,997)]

11. I understand that in the latest July 18, 2000 Office Action claims 459-472 and 474-575 were rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s) at the time the application was filed, had possession of the claimed invention. In this rejection, the Examiner indicated that "[s]upport was not found . . ." for the following limitations in claims 459-472 and 474-575:

Claims 459-463, specific chemical compositions of linkages;  
Claims 464-472, 482-569, specific identity of labels and points of attachment of the "SIG" moiety to internal phosphates;  
Claims 474-477 and 570-575, the "composition" limitation, in addition to the above identified limitations.

12. I understand that in the previous February 3, 1999 Office Action, claims 459-472 and 474-575 were also rejected for "new matter." In that Office Action (pages 2 and 3), the Examiner stated:

Support was not found where indicated in the specification, nor elsewhere, for the following limitation in Claims 459-472 and 474-575:

Claims 459-463, specific chemical composition of linkages;  
Claims 464-472, 482-569, specific identity of labels and points of attachment of the "SIG" moiety to internal phosphates;  
Claims 474-477 and 570-575, the "composition" limitation, in addition to the above identified limitations.

Applicant argues in the response, pages 30-32, that support was found in various portions of the specification. This argument is not persuasive. These portions merely recite support for covalent attachment of a SIG moiety to a phosphate moiety and a SIG moiety to the oxygen on a phosphate moiety. Note, there is no support for the attachment of the SIG moiety to the phosphate atom of the phosphate moiety, only to the oxygen atom.

13. I understand that in the July 18, 2000 Office Action, claims 454-575 were also rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention, reasons of record. In that Office Action (pages 3-4), the Examiner indicated:

Halloran et al., J. Immunol. 96(3):373-378, 1966 (Halloran) discloses the attachment of a specific signal moiety, a protein, to the phosphorus atom of the phosphate moiety using a specific linker, a -C-(CH<sub>2</sub>)<sub>4</sub>-N- chain. In contrast, the claims are drawn to a much broader

category, a generic "SIG" moiety and linkage, and specific compounds such as those recited in claim 464-magnetic, hormone, metal containing "SIG" moieties, for example.) Thus, the scope of the enablement is not commensurate with the scope of the claims.

In addition, several of the referenced articles are drawn to labeling a mononucleotide and express doubt about labeling an oligonucleotide. Armstrong et al., Eur. J. Biochem. 70:33-38, 1976, teaches that the labeled mononucleotides are "strong competitive inhibitors" of the reaction which is necessary to produce a labeled oligonucleotide from a labeled mononucleotide. See Armstrong, p. 33, col. 1. This reaction is the use of the labeled mononucleotide as a substrate for the polymerase mediated synthesis of the oligonucleotide. While Armstrong teaches that some labeled mononucleotide will be incorporated, Armstrong teaches no guidance as to which of the myriad labels within the scope of these claims will function in the claimed invention. Lacking any guidance in the specification and in view of the breadth of the claims, it would require undue experimentation in order to enable a reasonable number of embodiments of these claims. The skilled artisan would have to experiment with various SIG moieties, linkages and attachment sites, as well as various reaction protocols and protecting groups.

14. I understand that in the previous February 3, 1999 Office Action, claims 454-575 were also rejected for lack of enablement. In that Office Action (pages 3-4), the Examiner stated:

Applicant argues that the prior art, such as Halloran, supports enablement. This argument is not persuasive for several reasons. Halloran discloses the attachment of a specific signal moiety, a protein, to the phosphorus atom of the phosphate moiety using a specific linker, a -C-(CH<sub>2</sub>)<sub>4</sub>-N- chain. In contrast, the claims are drawn to a much broader category, a generic "SIG" moiety and linkage, and specific compounds such as those recited in claim 464 -- magnetic, hormone, metal containing "SIG" moieties, for example). Thus, the scope of the enablement is not commensurate with the scope of the claims.

In addition, several of the referenced articles are drawn to labeling a mononucleotide and express doubts about labeling an oligonucleotide. Armstrong et al., Eur. J. Biochem. 70:33-38, 1976, teaches that the labeled mononucleotides are "strong competitive inhibitors" of the reaction which is necessary to produce a labeled oligonucleotide from a labeled mononucleotide. See Armstrong, p. 33, col. 1. This reaction is the use of the labeled mononucleotide as a substrate for the polymerase mediated synthesis of the oligonucleotide.

While Armstrong teaches that some labeled mononucleotide will be incorporated, Armstrong teaches no guidance as to which of the myriad labels within the scope of the claims will function in the claimed invention. Lacking any guidance in the specification and in view of the breadth of the claims, it would require undue experimentation in order to enable a reasonable number of



Dean Engelhardt et al.

Serial No. 08/479,997

Filed: June 7, 1995

Page 11 [Declaration of Cheryl H. Agris, Ph.D., Attorney At Law (In Support of the Written Description, Enablement & Nonobviousness of the Invention Claimed in U.S. Patent Application Serial No. 08/479,997)]

embodiments of these claims. The skilled artisan would have to experiment with various SIG moieties, linkages and attachment sites, as well as various reaction protocols and protecting groups.

15. I further understand that in the July 18, 2000 Office Action, claims 454-575 were rejected under 35 U.S.C. §103 for being unpatentable over Halloran et al., J. Immunol. 96(3):373-378, 1966 or Miller et al., Biochemistry 20(7):1874-1880, 1981, for reasons of record. In the July 18, 2000 Office Action (page 4), the Examiner stated:

Both Halloran and Miller teach specific labels, (SIG moieties such as proteins and thiophosphates) attached to nucleic acids. See Halloran p. 373, Fig. 1 and col. 2; Miller p. 1874, col. 1. These prior art references differ from the claims in the recitation of some specific labels and linkages. It would have been *prima facie* obvious, however, to one of ordinary skill in the art at the time the invention was made to substitute any linker or label in the methods of Halloran and Miller for the expected benefit of constructing multiple labels. Given the fact that diverse labels such as proteins and thiophosphates, the ordinary artisan would have reasonably expected any moiety used as a label to function in the claimed invention.

On page 5 of the July 18, 2000 Office Action, the Examiner further stated:

Repeating an important point from a previous Office action, applicants arguments to the 35 U.S.C. §112, first paragraph rejections have provided strong evidence of obviousness and vice-versa. For example, the references used to support enablement, Halloran and Miller add evidence of obviousness.

It is important that the arguments for patentability explain, that the prior art supplied by applicant, for example, Halloran and Miller, can buttress the specification--providing needed evidence that the thin "SIG Phosphate" disclosure both "describes" and "enables" the detailed invention now claimed--but these same prior art references do not render the claims obvious.

Furthermore, the criticisms of the obviousness rejections must be made without undermining the enablement rejection. For example, if arguing that Halloran and Miller are somehow "non-enabled" one must justify how the specification can be enabled. After all, the prior art contains much more detail than that found in the specification.

Finally, the specification is held to a higher standard than the teachings of the prior art supplied to an obviousness rejection. As stated in a previous office action:

35 U.S.C. §112 provides that, in return for the grant of monopoly, the specification must enable one skilled in the art to "make and use" the invention "without undue experimentation" whereas U.S.C. §103 makes no such requirement. Thus, a teaching of how to use a compound can be entirely adequate to render a claim obvious but, at the same time, entirely inadequate to support the allowance of such a claim.

Dean Engelhardt et al.

Serial No. 08/479,997

Filed: June 7, 1995

Page 12 [Declaration of Cheryl H. Agris, Ph.D., Attorney At Law (In Support of the Written Description, Enablement & Nonobviousness of the Invention Claimed in U.S. Patent Application Serial No. 08/479,997)]

I also understand that the same rejection for obviousness was made in the previous February 3, 1999 Office Action.

16. As Enzo's consultant and on its behalf, I am making this Declaration in support of the adequate written description, enabling disclosure and non-obviousness of the claims which will be pending after submission of Applicants' Amendment Under 37 C.F.R. §1.116. To the extent that the subject matter is similar with those of the previously pending claims prior to submission of Applicants' Amendment, my remarks are applicable to those previously pending claims as well.

17. Based upon my own training, background and experience, I would submit that at the time this application was first filed in June 1982, a person of ordinary skill in the art relevant to the subject matter being claimed, including nucleic acid modification, synthesis, hybridization and detection, would have possessed or could have been actively pursuing an advanced degree in organic chemistry and/or biochemistry. Such an ordinarily skilled person could also be at least approaching or ranging toward the level of a junior faculty member with 2-5 years of relevant experience, or at least be a postdoctoral student with several years of experience. I consider myself to possess the level of skill and knowledge of at least a person of ordinary skill in the art to which the present application and invention pertains.

#### Written Description

18. As I understand it, the written description rejection concerns the following three issues:

- A. the specific chemical compositions of linkages recited in claims 459-463;
- B. the specific identity of labels and points of attachment of the "SIG" moiety to internal phosphates as recited in claims 464-472 and 482-569; and
- C. the composition limitations as recited in claims 474-477 and 570-575.

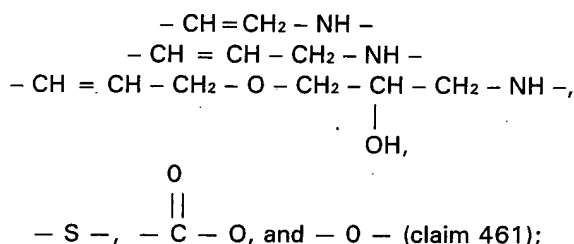
**A. Specific chemical compositions of linkages (claims 459-463)**

19. I understand that claims 459-463 are directed to subject matter where

the chemical linkage comprises a member selected from the group consisting of an olefinic bond at the  $\alpha$ -position relative to the point of attachment to the nucleotide, a  $-\text{CH}_2\text{NH}-$  moiety, or both (claim 459);

the chemical linkage comprises an allylamine group (claim 460);

the chemical linkage comprises or includes an olefinic bond at the  $\alpha$ -position relative to the point of attachment to the nucleotide, or any of the moieties:



the chemical linkage of Sig includes a glycosidic linkage moiety (claim 462); and

PM is a monophosphate, a diphosphate or a triphosphate and said Sig moiety is covalently attached to said PM through a phosphorus atom or phosphate oxygen (claim 463).

20. As a person skilled in the art to which the present invention pertains, I have reviewed the '997 specification as originally filed and it is my opinion and conclusion that the following portions of that disclosure support the above recited chemical linkages:

<u>Chemical Linkage(s)</u>	<u>Citation In Specification</u>	<u>Description in Disclosure</u>
olefinic bond at the $\alpha$ -position relative to the point of attachment to the nucleotide, a $-\text{CH}_2\text{NH}-$ moiety, or both	Page 11, 2nd ¶ Original Claim 78  Page 11, last ¶ Original Claim 79	that the chemical linkage include an olefinic bond at the $\alpha$ -position relative to B  that the chemical linkage group . . . have the structure $-\text{CH}_2\text{NH}-$ , . . .

<u>Chemical Linkage(s)</u>	<u>Citation In Specification</u>	<u>Description in Disclosure</u>
an allylamine group	Page 11, last ¶ Original Claim 80	Examples of preferred linkages derived from allylamine . . .
olefinic bond at the $\alpha$ -position relative to the point of attachment to the nucleotide or any of the moieties $\text{--CH=CH}_2\text{--NH--}$ $\text{--CH=CH--CH}_2\text{--NH--}$ $\text{--CH=CH--CH}_2\text{--O--CH}_2\text{--CH--CH}_2\text{--NH--}$ <div style="margin-left: 100px;">  OH</div>	Page 11, 2nd ¶   Page 11, line 29 Page 11, l. 29-30	preferred that the chemical linkage include an olefinic bond at the $\alpha$ -position relative to B. The presence of such an $\alpha$ -olefinic bond . . .   $\text{--CH=CH--CH}_2\text{--NH--}$ $\text{--CH=CH--CH}_2\text{--O--CH}_2\text{--CH--CH}_2\text{--NH--}$ <div style="margin-left: 100px;">  OH</div>
$\text{--S--}$	Original Claim 82	$\text{--S--}$
$\begin{array}{c} \text{O} \\    \\ \text{--C--O} \end{array}$	<i>ibid.</i>	$\begin{array}{c} \text{O} \\    \\ \text{--C--O} \end{array}$
$\text{--O--}$	<i>ibid.</i>	$\text{--O--}$
glycosidic linkage moiety	Original Claim 25	said Sig chemical moiety is attached by or includes a glycosidic linkage moiety.
PM is a monoP, diP triP and said Sig moiety is covalently attached to said PM through a phosphorus atom or phosphate oxygen	Page 9, lines 8-14   Page 57, Ex. V	wherein each of x, y and z represents $\text{H--}, \text{HO--}, \begin{array}{c} \text{O} \\    \\ \text{HO--P--O--} \end{array} \dots$ <div style="margin-left: 100px;">  OH</div> Biotin and polybiotinylated poly-L-lysine were coupled to oligoribonucleotides using a carbodiimide coupling procedure described by Halloran and Parker, <i>J. Immunol.</i> , <b>96</b> 373 (1966).

Thus, it is my opinion and conclusion as a person skilled in the art that the above-cited portions in the disclosure fully support the various chemical linkages recited in the pending claims of this application. The above-cited portions describe such recited chemical linkages in sufficient detail that one skilled in the art can reasonably conclude that the inventors had possession of such chemical linkages.

Dean Engelhardt et al.

Serial No. 08/479,997

Filed: June 7, 1995

Page 15 [Declaration of Cheryl H. Agris, Ph.D., Attorney At Law (In Support of the Written Description, Enablement & Nonobviousness of the Invention Claimed in U.S. Patent Application Serial No. 08/479,997)]

**B. Specific identity of labels and points of attachment of the "SIG" moiety to internal phosphates (claims 464-472 and 482-569)**

21. I understand that claims 464-472 and 482-567 (claims 568-569 having been canceled) are directed to subject matter for the following labels of the "SIG" moiety:

**Labels (SIG)**

**Claims 464, 492, 521 and 549** (Listed as (i) through (xvi) below)

- (i) biotin
- (ii) iminobiotin
- (iii) electron dense component
  - ferritin (claims 465, 493, 522 and 550)
- (iv) ligand and a specific ligand binding protein (claims 466, 494, 523 and 551 as amended)
- (v) magnetic component
  - magnetic oxide (claims 467, 495, 524 and 553)
  - ferric oxide (claims 468, 498, 525 and 552)
- (vi) enzyme or an enzyme component
  - alkaline phosphatase, acid phosphatase,  $\beta$ -galactosidase, ribonuclease, glucose oxidase and peroxidase (claims 469, 497, 526 and 554);
- (vii) hormone or a hormone component
- (viii) metal-containing component
  - catalytic (claims 470, 498, 527 and 555)
- (ix) fluorescent component
  - fluorescein, rhodamine and dansyl (claims 471, 499, 528 and 556)
- (x) chemiluminescent component
- (xi) antigen
- (xii) hapten
- (xiii) antibody or an antibody component
  - antigen or hapten capable of complexing with an antibody or antibody component specific thereto, and an antibody or antibody component capable of complexing with an

- antigen or hapten (claims 472, 500, 529 and 557);
- (xiv) composition comprising the oligo- or polydeoxyribonucleotide . . . , a polypeptide capable of forming a complex with Sig and a moiety which can be detected when such complex is formed (claims 502, 531 and 559); polypeptide comprises polylysine (claims 503, 532 and 560); polypeptide is selected from the group consisting of avidin, streptavidin and anti-hapten immunoglobulin (claims 504, 533 and 561);
- (xv) composition of . . . , wherein Sig is a ligand and said polypeptide is an antibody thereto (claims 505, 534 and 562); and
- (xvi) Sig is or renders the nucleotide or the oligo- or polynucleotide self-signaling or self-indicating or self-detecting (claims 512 and 540).

22. I have reviewed the '997 specification as originally filed and it is my opinion and conclusion as a skilled person in the art that the following portions of that disclosure support the above recited labels or SIG listed as items (i) through (xvi):<sup>2</sup>

<u>Label (Sig)</u>	<u>Citation In Specification</u>	<u>Description in Disclosure</u>
biotin	Page 10, last 2 lines Page 97, last ¶, thru Page 98, 1st 4 lines Original Claim 92	. . . the preferred A moieties are biotin and iminobiotin. . . . the chemical moiety A . . . is functionally the equivalent of the Sig component or chemical moiety . . . of this invention. . . Sig chemical moiety is biotin
iminobiotin	<i>ibid.</i>	
electron dense component	Page 97, 1st ¶	The Sig moiety might also include an electron dense component, . . .

<sup>2</sup> The citations and descriptions listed below are not necessarily intended to be exhaustive of all the support for any given label or Sig. Rather, the citations and descriptions are offered as illustrative support which is non-limiting.

<u>Label (Sig)</u>	<u>Citation In Specification</u>	<u>Description in Disclosure</u>
ferritin	<i>ibid.</i>	such as ferritin, . . .
ligand and a specific binding protein	Page 101, thru Page 102	. . . presence of probe is assayed for by the specific binding of the protein to the ligand . . .
magnetic component	Page 97, 1st ¶	magnetic component associated or attached thereto, . . .
magnetic oxide	<i>ibid.</i>	such as a magnetic oxide,
ferric oxide	<i>ibid.</i>	or magnetic iron oxide, . . .
enzyme or an enzyme component:	Page 96, 2nd ¶	The Sig moiety could comprise an enzyme or enzymic material
alkaline phosphatase	<i>ibid.</i>	such alkaline phosphatase,
acid phosphatase	Original Claim 41 Original Claim 197	said enzyme is acid phosphatase group . . . acid phosphatase . . .
$\beta$ -galactosidase	Page 36, 3rd ¶ Also Original Claim 84	direct enzymes such as . . . or $\beta$ -galactosidase . . . said enzyme is $\beta$ -galactosidase.
ribonuclease	Page 96, 2nd ¶	or ribonuclease.
glucose oxidase	<i>ibid.</i>	glucose oxidase,
peroxidase	<i>ibid.</i>	horseradish peroxidase,
hormone or a hormone component	Page 102, 1st ¶	3. Hormone receptors and other receptors on the surface of the cell . . .
metal-containing component	Original Claim 28	. . . metal-containing component
catalytic	Original Claim 83 Original Claim 174	said Sig chemical moiety includes or comprises a catalytic metal component . . . catalytically active metal.
fluorescent component	Page 96, 1st ¶	The Sig moiety could include a fluorescing component
fluorescein, rhodamine or dansyl	<i>ibid.</i>	such as fluorescein or rhodamine or dansyl.

Dean Engelhardt et al.

Serial No. 08/479,997

Filed: June 7, 1995

Page 18 [Declaration of Cheryl H. Agris, Ph.D., Attorney At Law (In Support of the Written Description, Enablement & Nonobviousness of the Invention Claimed in U.S. Patent Application Serial No. 08/479,997)]

<u>Label (Sig)</u>	<u>Citation In Specification</u>	<u>Description in Disclosure</u>
chemiluminescent component	Page 97, 1st ¶	The Sig component or moiety could include . . . a chemiluminescent component
antigen	Original Claim 28 See also Page 88, 2nd ¶	an antigen . . . fix to a solid matrix a specific antigen and bind to this antigen an antibody directed against this antigen which itself has been biotinylated.
hapten	Page 97, 1st ¶	could include a hapten component
antibody or an antibody component	Original Claim 28	or antibody component.
antigen or hapten capable of complexing with antibody . . .	Original Claim 136	said Sig chemical moiety includes an antigenic or hapten component capable of complexing with an antibody specific to said component.
antibody or an antibody component capable of complexing with an antigen or hapten	<i>ibid.</i> See also Page 88, last ¶	The use of the antigen-antibody system for detecting either antigen or antibody is well known.
composition . . . oligo- or polynucleotide . . . polypeptide and moiety which can be detected	Original Claims 167 & 168	A polynucleotide comprising one or more nucleotides . . . coupled to a polypeptide, . . . having attached . . . one or more Sig chemical moieties
polypeptide . . . polylysine	Original Claim 56	said polypeptide is a polylysine.
polypeptide . . . avidin, streptavidin and anti-hapten immunoglobulin	Page 25, last 2 lines Page 26, 3rd ¶  Page 34, 1st ¶	One polypeptide detector for the biotinyl-type probe is avidin. A preferred probe for biotin-containing nucleotides and derivatives is streptavidin, an avidin-like protein . . . We can haptenize this secondary antibody with biotin and thus generate two anti-hapten IgG populations, . . .



Dean Engelhardt et al.

Serial No. 08/479,997

Filed: June 7, 1995

Page 19 [Declaration of Cheryl H. Agris, Ph.D., Attorney At Law (In Support of the Written Description, Enablement & Nonobviousness of the Invention Claimed in U.S. Patent Application Serial No. 08/479,997)]

<u>Label (Sig)</u>	<u>Citation In Specification</u>	<u>Description in Disclosure</u>
Sig is a ligand and said polypeptide is an antibody	Page 101, 1st ¶	. . presence of probe is assayed for by the specific binding of the protein to the ligand . . .
self-signaling or self-indicating or self-detecting	Page 82, 1st ¶	Of special importance and significance . . . self-signaling or self-indicating or self-detecting nucleic acids,

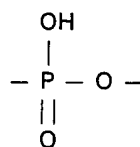
Thus, it is my opinion and conclusion as a person skilled in this art that the above-cited portions in the disclosure fully support the various labels which embody Sig in the pending claims of this application. The above-cited portions which I find to be in general quite explicit describe the claimed labels or Sig in sufficient detail that one skilled in the art can reasonably conclude that the inventors had possession of such claimed labels or Sig.

## 2. Attachment of SIG to Internal Phosphates

23. I understand that among claims 464-472 and 482-567 (claims 568-569 having been canceled), certain of these are directed to the points of attachment of the "SIG" moiety to internal phosphates:

An oligo- or polydeoxyribonucleotide comprising at least one modified nucleotide having the structural formula . . . wherein x, y and z are selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate and wherein Sig is covalently attached directly or through a chemical linkage to at least one phosphate selected from the group consisting of x, y, z, and a combination thereof . . . (claim 482);

oligo- or polydeoxyribonucleotide of . . . wherein said covalent attachment is selected from . . .



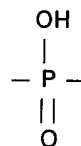
and

Dean Engelhardt et al.

Serial No. 08/479,997

Filed: June 7, 1995

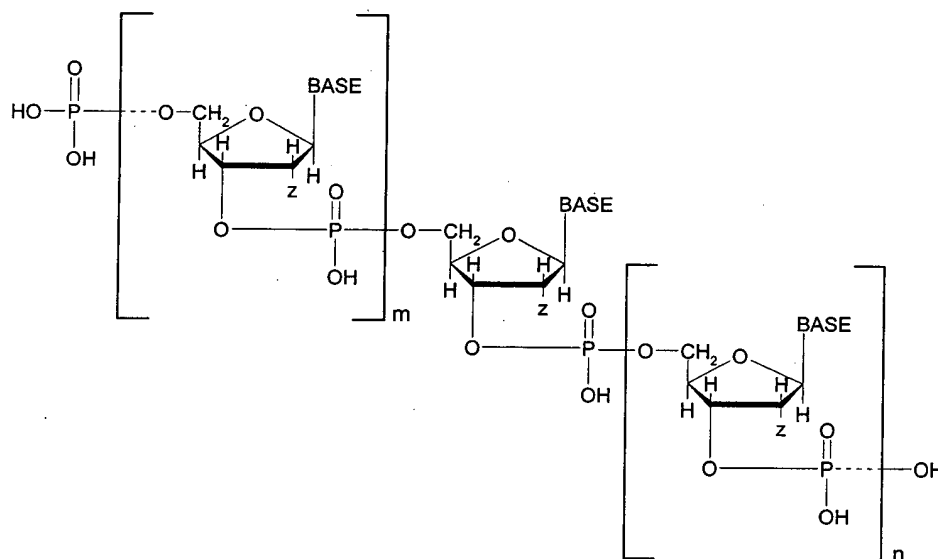
Page 20 [Declaration of Cheryl H. Agris, Ph.D., Attorney At Law (In Support of the Written Description, Enablement & Nonobviousness of the Invention Claimed in U.S. Patent Application Serial No. 08/479,997)]



(claims 485, 514 and 542)

oligo- or polydeoxyribonucleotide of . . . wherein said x and y each comprise a member selected from the group consisting of mono-, di or tri-phosphate and said Sig moiety is covalently attached to either or both of said x and y through a phosphorus or phosphate oxygen(claim 491, 520 and 548);

oligo- or polydexoyribonucleotide of . . . having the structural formula:

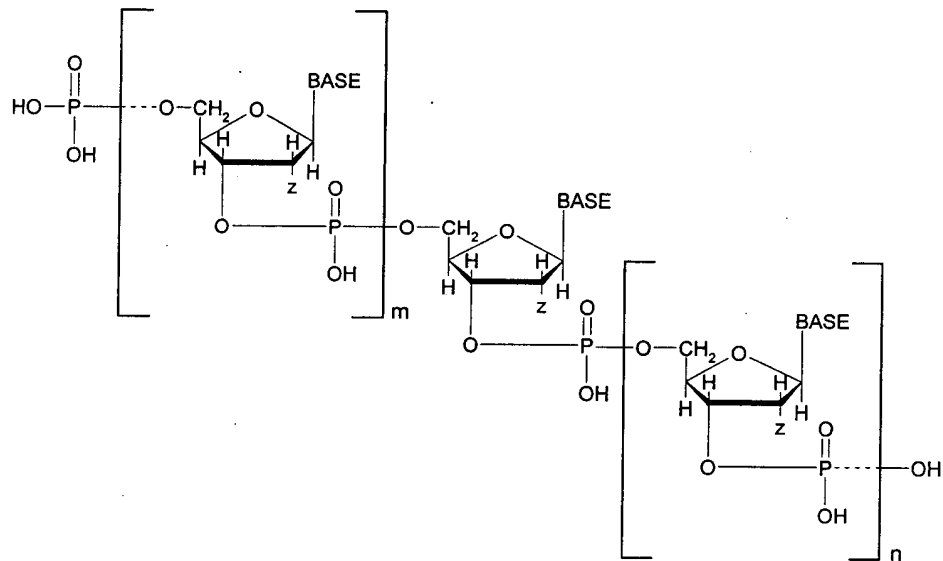


wherein said Sig moiety is attached to at least one of the phosphate moieties in said structural formula (claim 510);

oligo- or polynucleotide . . . having the structural formula Sig - PM - SM - BASE . . . Sig is covalently attached to PM directly or via a chemical linkage (claim 511) [recited in Paragraph 10C above];

oligo- or polyribonucleotide comprising at least one nucleotide having the structural formula . . . Sig is covalently attached to x, y or z directly or through a chemical linkage (claim 539) [recited in Paragraph 10D above]; and

oligo- or polyribonucleotide of . . ., having the structural formula:



25. As a patent practitioner, I have lectured and written on the requirements of 35 U.S.C. § 112, including the Written Description requirements under the first paragraph of § 112, and I have also submitted comments on the Interim Written Description Guidelines issued July 7, 1998, 63 FR 32,639. I wish to point out that Applicants' disclosure and their claimed subject matter also meets the January 5, 2001 Written Description Guidelines (Exhibit 7), particularly with respect to the attachment of Sig to the phosphorus atom of the phosphate moiety. Those guidelines provide that the written description requirement is met when the patent specification describes the claimed invention in "sufficient detail that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention" (citing *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 19 USPQ2d 1111 (Fed. Cir. 1991) 66 FR 1099 (2001)). According to these guidelines, possession may be shown by showing that the invention was "ready for patenting" such as by

the disclosure of drawings or other descriptions of the invention that are sufficiently specific to enable a person skilled in the art to practice the invention. *Id.* It is well established case law that when the elements recited in the claims are supported by corresponding language in the text, examples, drawings, or other disclosure in the specification, the written description requirement is satisfied and no further analysis is required. *In re Bowen*, 492 F.2d 859 (C.C.P.A. 1974). Furthermore, if new claims are proposed during prosecution, each claim must be expressly, implicitly or inherently supported in the originally filed disclosure and each claim must include all elements which applicant has described as essential. 66 FR at 1105. To establish inherency, it must be clear from any extrinsic evidence provided in the missing descriptive matter is necessarily present in the thing described in the reference and that it would be so recognized by persons of ordinary skill. *In re Robertson*, 169 F.3d 743, 49 USPQ2d 1949 (Fed. Cir. 1999).

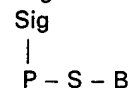
26. I would also point out that training materials have also been provided in connection with the Revised Interim Written Description Guidelines issued on December 21, 1999. A decision tree was included with those training materials and is attached to my Declaration as Exhibit 8. Although revisions are expected to the training materials in view of the final Written Description Guidelines, these training materials appear to be still in effect. 66 FR at 1099. When a claim of broader scope is added, the question posed is "Is an element(s) missing from the claim?" If the answer is "yes", the question posed is "Is the missing element(s) described by applicant as being an essential or critical feature of the new claim as a whole?" If the answer is "no", the question posed is "Is there express, inherent or implicit support for the claim as a whole?" If the answer is "yes", the written description requirement is met.

27. For reasons explained in the succeeding subsections of this paragraph (A-D), it is my opinion and conclusion that the written description requirement has been met.

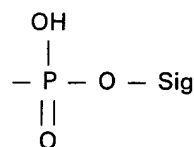
A. First, several structures are depicted and descriptions given where "Sig" is attached to the phosphate moiety. As described on pages 8-10 in the Engelhardt Declaration (Exhibit 6), such structures are found variously in the specification, for example, on page 94, last paragraph, and continuing through

page 95, first paragraph:

Still further, nucleotides in accordance with the practices of this invention include the nucleotides having the formula



wherein P is the phosphoric acid moiety, S the sugar moiety and B the base moiety. In these special nucleotides the P moiety is attached to the 3' and/or the 5' position of the S moiety when the nucleotide is deoxyribonucleotide and at the 2', 3' and/or 5' position when the nucleotide is a ribonucleotide. The base B is either a purine or a pyrimidine and the B moiety is attached from the N1 or the N9 position to the 1' position of the sugar moiety when said B moiety is a pyrimidine or a purine, respectively. The Sig chemical moiety is covalently attached to the phosphoric acid P moiety via the chemical linkage



said Sig, when attached to said P moiety being capable of signalling itself or making itself self-detecting or its presence known and desirably the nucleotide is capable of being incorporated into a double-stranded or DNA, RNA or DNA-RNA hybrid.

Later in the specification, on page 96, and continuing through the first paragraph on page 98, other descriptions are provided wherein Sig is attached to the phosphate moiety:

By way of summary, as indicated hereinabove with respect to the make-up of the various special nucleotides in accordance with this invention, the special nucleotides can be described as comprising a phosphoric acid moiety P, a sugar moiety S and a base moiety B, a purine or pyrimidine, which combination of P-S-B is well known with respect to and defines nucleotides, both deoxyribonucleotides and ribonucleotides. The nucleotides are then modified in accordance with the practices of this invention by having covalently attached thereto, to the P moiety and/or the S moiety and/or the B moiety, a chemical moiety Sig. The chemical moiety Sig so attached to the nucleotide P-S-B is capable of rendering or making the resulting nucleotide, now comprising P-S-B with the Sig moiety attached to one or more of the other moieties, self-detecting or signalling itself or capable of making its presence known per se, when incorporated into a polynucleotide, especially a double-stranded polynucleotide, such as double-stranded DNA, a double-stranded RNA or a double-stranded DNA-RNA hybrid. The Sig moiety desirably should not interfere with the capability of the nucleotide to form a double-stranded polynucleotide containing the special Sig-containing nucleotide in accordance with this invention and, when so incorporated therein, the Sig-containing nucleotide is capable of detection, localization or observation.

The Sig moiety employed in the make-up of the special nucleotides of this invention could comprise an enzyme or enzymic material, such as alkaline phosphatase, glucose oxidase, horseradish peroxidase or ribonuclease. The Sig moiety could also contain a fluorescing component, such as fluorescein or rhodamine or dansyl. If desired, the Sig moiety could include a magnetic component associated or attached thereto, such as a magnetic oxide or magnetic iron oxide, which would make the nucleotide or polynucleotide containing such a magnetic-containing Sig moiety detectable by magnetic means. The Sig moiety might also include an electron dense component, such as ferritin, so as to be available by observation. The Sig moiety could also include a radioactive isotope component, such as radioactive cobalt, making the resulting nucleotide observable by radiation detecting means. The Sig moiety could also include a hapten component or per se be capable of complexing with an antibody specific thereto. Most usefully, the Sig moiety is a polysaccharide or oligosaccharide or monosaccharide, which is capable of complexing with or being attached to a sugar or polysaccharide binding protein, such as a lectin, e.g. Concanavilin A. The Sig component or moiety of the special nucleotides in accordance with this invention could also include a chemiluminescent component.

As indicated in accordance with the practices of this invention, the Sig component could comprise any chemical moiety which is attachable either directly or through a chemical linkage or linker arm to the nucleotide, such as the base B component therein, or the sugar S component therein, or the phosphoric acid P component thereof.

The Sig component of the nucleotides in accordance with this invention and the nucleotides and polynucleotides incorporating the nucleotides of this invention containing the Sig component are equivalent to and useful for the same purposes as the nucleotides described in the above-identified U.S. patent application Serial No. 255,223. More specifically, the chemical moiety A described in U.S. patent application Serial No. 255,223 is functionally the equivalent of the Sig component or chemical moiety of the special nucleotides of this invention. Accordingly, the Sig component or chemical moiety of nucleotides of this invention can be directly covalently attached to the P, S or B moieties or attached thereto via a chemical linkage or linkage arm as described in U.S. patent application Ser. No. 255,223, as indicated by the dotted line connecting B and A of the nucleotides of U.S. Serial No. 255,223. The various linker arms or linkages identified in U.S. Ser. No. 255,223 are applicable to and useful in the preparation of the special nucleotides of this invention.

B. Furthermore, as set forth on pages 10-11 of the Engelhardt Declaration (Exhibit 6), there are nine separate instances where Sig is described in the specification as being attached to the phosphate moiety P (as well as the sugar moiety S and/or the base moiety B):

Dean Engelhardt et al.

Serial No. 08/479,997

Filed: June 7, 1995

Page 25 [Declaration of Cheryl H. Agris, Ph.D., Attorney At Law (In Support of the Written Description, Enablement & Nonobviousness of the Invention Claimed in U.S. Patent Application Serial No. 08/479,997)]

<u>Specification</u>	<u>Description</u>
page 90, last paragraph	. . . and a signalling chemical moiety Sig covalently attached thereto, either to the P, S or B moiety.
page 93, first paragraph	. . . include a chemical moiety Sig covalently attached to the P, S and/or B moieties.
page 96, first paragraph	. . . by having covalently attached thereto, to the P moiety and/or the S moiety and/or the B moiety, a chemical moiety Sig.
page 98, first paragraph	. . . the Sig component or chemical moiety of nucleotides of this invention can be directly covalently attached to the P, S or B moieties or attached thereto via a chemical linkage or linkage arm . . .
page 103, first full paragraph	. . . and the signalling or self-detecting moiety, Sig, covalently attached to either the P, S or B moieties, as indicated hereinabove, . . .
page 104, first paragraph	. . . nucleotides in accordance with this invention containing the above-described components P, S, B and Sig . . .
page 105, first paragraph	. . . the nucleotides of this invention include the P, S, B and Sig components wherein the Sig is covalently attached to either the P, S or B moieties
page 105, second paragraph	The moiety Sig attached to the special nucleotides of this invention containing the other moieties or components P, S, B provide a site per se for the attachment thereto, the Sig component, . . .
page 106, first paragraph	. . . the special P, S, B and Sig-containing nucleotides of this invention, . . .

Upon seeing such structures and their descriptions in the specification, one of ordinary skill in the art would clearly understand that "Sig" may be attached to either the oxygen or phosphorus atom of the phosphate moiety. As described in the Engelhardt Declaration (Exhibit 6), methods are provided in the specification and are well known in the art for attaching a substituent to the phosphorus and to the oxygen atom. Therefore, in my opinion, the written description requirement has been met.

Dean Engelhardt et al.

Serial No. 08/479,997

Filed: June 7, 1995

Page 26 [Declaration of Cheryl H. Agris, Ph.D., Attorney At Law (In Support of the Written Description, Enablement & Nonobviousness of the Invention Claimed in U.S. Patent Application Serial No. 08/479,997)]

C. Thirdly, I hold the same opinion and I have reached the same conclusion in applying the analysis set forth in the decision tree in the training materials (Exhibit 8) provided in connection with the Written Description Guidelines. As a person of ordinary skill in the art, I also recognize that the scope of claims 459-472 and 474-567 is somewhat broader than the original claims. Specifically, the originally filed claims (see, for example, original claims 141) contained the requirement that "Sig" be attached to the oxygen atom in the phosphate moiety (PM). The currently pending claims do not contain such a requirement. This missing element is not an essential or critical feature of the new claims as a whole because there is no specific requirement set forth in the specification that "Sig" be attached to the oxygen atom in the phosphate moiety.

D. Further, in my opinion and conclusion, there is inherent support for the subject matter of claims 459-474 and 474-567. As a person of ordinary skill in the art, it is my opinion and conclusion that a reading of the specification, including Example V and the extrinsic evidence detailed in the Engelhardt Declaration (Exhibit 6), reasonably conveys that in accordance with the present invention, when a substituent is depicted as being attached to a phosphate moiety (PM), that such a substituent could be attached to either an oxygen or phosphorus atom.

Accordingly, it is my opinion and conclusion that Applicants' claimed subject matter wherein the Sig moiety is attached to the phosphorus or oxygen atom of the phosphate moiety meets the requirements for written description.

**C. Composition limitations (claims 474-477 and 570-575)**

28. In view of the cancellation of claims 570-575 to be effected by Applicants' Amendment Under 37 C.F.R. §1.116, my remarks below are directed to claims 474-477. These last-mentioned claims contain the following subject matter:

a composition comprising the oligo- or polydeoxyribonucleotide of claim 454, a polypeptide capable of forming a complex with Sig and a moiety which can be detected when such complex is formed (claim 474);

the composition . . . wherein said polypeptide comprises polylysine (claim 475);



Dean Engelhardt et al.

Serial No. 08/479,997

Filed: June 7, 1995

Page 27 [Declaration of Cheryl H. Agris, Ph.D., Attorney At Law (In Support of the Written Description, Enablement & Nonobviousness of the Invention Claimed in U.S. Patent Application Serial No. 08/479,997)]

the composition . . . wherein said polypeptide is selected from the group consisting of avidin, streptavidin and anti-Sig immunoglobulin (claim 476); and

the composition . . . wherein Sig is a ligand and said polypeptide is an antibody thereto (claim 477).

29. I have reviewed the '997 specification as originally filed and it is my opinion and conclusion as a skilled person in the art that the following portions of that disclosure support the above recited compositions:

<u>Composition(s)</u>	<u>Citation In Specification</u>	<u>Description in Disclosure</u>
comprising oligo- or polydeoxyribonucleotide . . . , a polypeptide capable of forming a complex with Sig and a moiety which can be detected when such complex is formed	Page 8, last ¶  Page 97, last ¶, thru Page 98, 1st 4 lines	wherein A represents a moiety . . . which is capable of forming a detectable complex with a polypeptide . . . the chemical moiety A described in U.S. patent application Serial No. 255,223 is functionally the equivalent of the Sig component or chemical moiety . . . of this invention.
wherein said polypeptide comprises polylysine	Original Claim 56	said polypeptide is a polylysine.
polypeptide . . . avidin, streptavidin and anti-hapten immunoglobulin	Page 25, last 2 lines Page 26, 3rd ¶  Page 34, 1st ¶	One polypeptide detector for the biotinyl-type probe is avidin. A preferred probe for biotin-containing nucleotides and derivatives is streptavidin, an avidin-like protein . . . We can haptenize this secondary antibody with biotin and thus generate two anti-hapten IgG populations, . . .
Sig is a ligand and said polypeptide is an antibody	Page 101, 1st ¶	. . . presence of probe is assayed for by the specific binding of the protein to the ligand . . .

30. Based upon the above-cited portions, it is my opinion and conclusion as a person skilled in the art that the specification as originally filed fully supports the compositional subject matter in claims 474-477. I find that the disclosure provides sufficient detail that a person skilled in the art can reasonably conclude that the inventors had possession of the subject matter in claims 474-477 at the time the

application was originally filed in June 1982.

***Enablement***

31. As a person of at least ordinary skill in the art to which the present invention pertains, it is my opinion and conclusion that the original disclosure of the '997 specification was enabling and permitted the practice of the subject matter of claims 454-567 without undue experimentation. My reasons for concluding that the '997 specification is enabling are set forth below.

32. First, although Halloran et al. only teaches one moiety, other moieties are disclosed in the specification and are known in the art. Specifically, Example V of the instant specification discloses a method for attaching biotin, one of the embodiments for Sig, to the phosphate moiety of a mononucleotide and an oligonucleotide that are coupled to a protein, poly-L-lysine. Furthermore, as detailed on pages 11 and 12 in the Engelhardt Declaration (Exhibit 6), the chemistry and reactions for attaching substituents to the oxygen or phosphorus atoms in a nucleotidyl phosphate or phosphoric acid moiety were already known in the art at the time the initial application was filed in June 1982.

33. Second, I would like to respond to statements made in the July 18, 2000 Office Action regarding the Armstrong et al. reference. Specifically, it is stated on page 3 in that Office Action that:

Armstrong et al., Eur. J. Biochem. 70:33-38, 1976, teaches that the labeled mononucleotides are "strong competitive inhibitors" of the reaction which is necessary to produce a labeled oligonucleotide from a labeled mononucleotide. See Armstrong, p. 33, col. 1. This reaction is the use of the labeled mononucleotide as a substrate for the polymerase mediated synthesis of the oligonucleotide.

The conclusion is reached in the Office Action that:

. . . Lacking any guidance in the specification and in view of the breadth of the claims, it would require undue experimentation in order to enable a reasonable number of embodiments of these claims. The skilled artisan would have to experiment with various SIG moieties, linkages and attachment sites, as well as various reaction protocols and protecting groups.

Dean Engelhardt et al.

Serial No. 08/479,997

Filed: June 7, 1995

Page 29 [Declaration of Cheryl H. Agris, Ph.D., Attorney At Law (In Support of the Written Description, Enablement & Nonobviousness of the Invention Claimed in U.S. Patent Application Serial No. 08/479,997)]

In response, it is my opinion and conclusion as a person skilled in the art that the labeled mononucleotides referenced in Armstrong et al. were strong competitive inhibitors because unmodified nucleoside triphosphates (NTPs) were present in the assay mixture. This would not be the case or a factor in the present invention. Armstrong et al. actually show that it is possible to incorporate such modified mononucleotides and that modified NTPs may be used as substrates. Although Armstrong et al. does disclose that some modified nucleotides are better than others in terms of binding to RNA polymerase, a person skilled in the art would expect that some routine testing or refinement is necessary.

#### ***Non-Obviousness***

34. As a person of at least ordinary skill in the art to which the present invention pertains, it is my opinion and conclusion that the claimed subject matter at hand in the form of claims 454-567 would not have been obvious at the time the invention was made from a reading of either Halloran et al. [*J. Immuno.* 96(3):373-378 (1966)] or Miller et al. [*Biochemistry* 20(7):1874-1880 (1981)]. My reasons for the non-obviousness of the invention of claims 454-567 are set forth in the paragraphs below.

35. It is asserted in the last Office Actions that both Halloran et al. and Miller et al. teach specific labels. Therefore, it would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to substitute any linker or label in the methods of Halloran and Miller for the expected benefit of constructing multiple labels. In paragraph 11 of the February 3, 1999 Office Action, it is requested that arguments for patentability explain that the prior art supplied by applicant can buttress the specification providing needed evidence that the "SIG-phosphate" disclosure both "describes" and "enables" the detailed invention now claimed, but that these same prior art references do not render the claims obvious.

36. As one skilled in the art, it is my opinion and conclusion that neither Halloran et al. nor Miller et al. actually disclose or suggest a "Sig-phosphate" moiety as defined in the Serial No. 08/479,997 specification. Specifically, Halloran et al. is

Dean Engelhardt et al.

Serial No. 08/479,997

Filed: June 7, 1995

Page 30 [Declaration of Cheryl H. Agris, Ph.D., Attorney At Law (In Support of the Written Description, Enablement & Nonobviousness of the Invention Claimed in U.S. Patent Application Serial No. 08/479,997)]

directed to covalently conjugating polynucleotides to proteins as a means of stimulating antibody formation. Miller et al. is directed to non-ionic oligonucleotide analogs. These analogs may be more easily taken up by cells than oligodeoxyribonucleotides and are resistant to cleavage by a variety of nucleases. Neither of these modified polynucleotides were ever made for labeling purposes, nor were such use of Halloran's or Miller's modified polynucleotides ever suggested by the cited documents. Certainly, neither the protein in Halloran et al. nor the methyl group in Miller et al. would be considered a detectable label as defined in the art or a detectable Sig chemical moiety as defined in the '997 specification. Before the advent of the invention now claimed, there had been no suggestion regarding attaching a Sig group, that is, a moiety capable of non-radioactive detection when attached to the phosphate moiety of at least one nucleotide in an oligo- or polydeoxyribonucleotide or polynucleotide.

37. As one skilled in the art to which this invention pertains, I wish to point out that there had been teachings regarding modifying the phosphate moiety with various substituents, but none of these substituents comprised a Sig-phosphate as set forth in the present claims. Once such a concept for modifying the phosphate moiety of a nucleotide was formulated, one of ordinary skill in the art would have looked to prior art references in order to develop methods for obtaining the oligo- or poly(deoxyribo)nucleotides of the present invention. As a specific example, Halloran et al. disclosed methods for coupling proteins to nucleotides and oligonucleotides. This reference may be used as a general reference for coupling amino acid moieties to the phosphate moiety. There is no disclosure in Halloran et al., however, that a final oligo- or poly(deoxyribo)nucleotide comprising at least one modified nucleotide of the formula set forth in the pending claims could be obtained.

38. With respect to the cited Miller reference, I am very familiar with the chemistry employed to make oligonucleoside methylphosphonates, having been a graduate student in Dr. Paul Miller's laboratory during the years 1979-1986. During this time, I and other members of Dr. Miller's group looked to procedures published on obtaining oligonucleotides in order to synthesize oligonucleoside methylphosphonates. For example, the condensing agent used in the cited Miller et al. reference, mesitylene sulfonyl tetrazolide, was used in synthesizing

Dean Engelhardt et al.

Serial No. 08/479,997

Filed: June 7, 1995

Page 31 [Declaration of Cheryl H. Agris, Ph.D., Attorney At Law (In Support of the Written Description, Enablement & Nonobviousness of the Invention Claimed in U.S. Patent Application Serial No. 08/479,997)]

oligonucleotides. A copy of Stawinski et al. [Nucleic Acids Research 4:353-371 (1977)] which describe the use of mesitylene sulfonyl tetrazolide in synthesizing oligonucleotides is attached as Exhibit 9. Also attached is section II.A. of my Ph.D. thesis (Exhibit 2) detailing strategies used in synthesizing oligonucleoside methylphosphonates. Although the thesis was not submitted until January 1986, the synthetic work described on pages 13-16 actually took place between 1979-1983. I was personally involved in synthesizing oligonucleoside methylphosphonates in June 1982. As described in these pages, methods known in the art for synthesizing oligodeoxyribonucleotides were used as a basis for synthesizing oligonucleoside methylphosphonates. However, neither I nor in my opinion, others of ordinary skill in the art had a reasonable expectation of success that these procedures could be successfully used in preparing oligonucleoside methyl phosphonates. For example, Stawinski et al. (Exhibit 9) merely provided a starting point regarding possible reaction conditions that could be used. There is no suggestion in Stawinski et al., however, that arylsulfonyltetrazoles could or even should be used to synthesize oligonucleoside methylphosphonates. The references cited were primarily used as guidelines for trying to formulate methods for synthesizing oligonucleoside methylphosphonates.

39. Similarly, it appears that the Miller and Halloran references were used as guidelines in formulating the oligo- or poly(deoxyribo)nucleotides of the present invention. The disclosures in each of these references would be sufficiently enabling for one of ordinary skill in the art for formulating procedures for synthesizing the oligo- or poly(deoxy)ribonucleotides of the present invention. There was no suggestion, however, that these procedures could or should be used to obtain, nor would one of ordinary skill in the art have a reasonable expectation of success in obtaining the oligo- or poly(deoxy)ribonucleotides of the present invention. Thus, it is my opinion and conclusion that a person of ordinary skill in the art would not have arrived at the invention claimed in U.S. Patent Serial No. 08/479,995 from a reading of the cited Miller or Halloran references, or even by combining the two references.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false

Dean Engelhardt et al.

Serial No. 08/479,997

Filed: June 7, 1995

Page 32 [Declaration of Cheryl H. Agris, Ph.D., Attorney At Law (In Support of the Written Description, Enablement & Nonobviousness of the Invention Claimed in U.S. Patent Application Serial No. 08/479,997)]

statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that any such willful false statements may jeopardize the validity of the application or any patent issued thereon.

11/7/01  
Date

Cheryl H. Agris  
Cheryl H. Agris, Ph.D.  
Attorney At Law

\* \* \* \* \*

FinalDraftDeclaration.CHA.1.16.01

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### **DEGREE PROGRAMS:**

- 1992: J.D., Brooklyn Law School  
Top 15%
- 1986: Ph.D., The Johns Hopkins University, Baltimore, Maryland  
School of Hygiene and Public Health  
Department of Biochemistry, Division of Biophysics  
Thesis: "Effects of Oligonucleoside Methylphosphonates on  
Vesicular Stomatitis Virus Protein Synthesis and Infection"
- 1979: Bachelor of Arts, Chemistry, Goucher College,  
Towson, Maryland  
Cumulative grade point average: 3.57/4.00

### **CONTINUING EDUCATION:**

- 2000: NASD Arbitrator Training Program; Writing and Using Intellectual Property  
Opinions (Association of the Bar of the City of New York); International  
Intellectual Property Symposium, Brooklyn Law School; Preparing Legal Opinions  
1999: Intellectual Property Due Diligence in Business Transactions (Association of  
the Bar of the City of New York)
- 1998: New York, New Jersey, Connecticut, and Pennsylvania Joint Seminar on  
Developments in Patent Law
- 1996: The Basics of Licensing and Licensing Law (The Licensing Journal)
- 1995: Patent Aspects of GATT (ABA, Section of Intellectual Property Law)  
Advanced PCT Practice (Patent Resources Group)
- 1994: European Patent Office Practice (Patent Resources Group)
- 1993: Technology Licensing and Litigation (Practicing Law Institute)

### **ADMISSIONS:**

New York and New Jersey State Bars  
Registered to practice before the U.S. Patent and Trademark Office

## **LEGAL EXPERIENCE:**

- 1998-present: Solo practitioner  
Preparation and prosecution of U.S. patent applications in the biotechnology, pharmaceutical and chemical fields; overseeing foreign prosecution of patent applications; patentability and validity studies; infringement analysis; freedom of operation studies; preparation of licensing, consulting and confidentiality agreements; peer review of patent prosecution by third parties; arbitrator
- 1992-1998: Patent Attorney, Novo Nordisk of North America, N.Y., N.Y.  
Preparation and prosecution of U.S. patent applications in the biotechnology and chemical fields; prosecution of foreign applications in the United States in the biotechnology, chemical, and pharmaceutical fields; supervising foreign filings; supervising patent liaison in California subsidiary; patentability and validity studies; infringement analysis; and preparation of licensing, consulting, confidentiality, and research agreements
- 1988-1992: Pennie & Edmonds, Law Clerk, Biotechnology Group  
Preparation and prosecution of U.S. patent applications in the biotechnology, chemical and pharmaceutical fields and interactions with foreign associates regarding foreign prosecution; assisted in patentability studies, validity studies, and infringement analyses and assisted in the preparation of consulting, confidentiality and licensing agreements

## **PRE-LEGAL EXPERIENCE:**

- 1986 -1988: Research Fellow, Sloan Kettering Institute  
Investigated the mechanism of the block in splicing of influenza viral NS1 mRNA to NS2 mRNA in vitro using molecular biological and biochemical approaches
- 1979-1986: Predoctoral Fellow, Johns Hopkins University  
Formulated methods for synthesizing the antisense nonionic oligonucleotide analogs, oligonucleoside methylphosphonates; studied the effects of oligonucleoside methylphosphonate sequences on the synthesis of VSV (vesicular stomatitis virus) proteins in cell culture and in vitro
- 1979: Undergraduate Research Associate, Argonne National Laboratory.  
Analyzed bile acids isolated from the bile, urine, or serum from children with cholestatic liver disease using gas chromatography and gas chromatography/mass spectroscopy

## **HONORS, AWARDS, AND FELLOWSHIPS:**

- 1988-1992: Richardson Scholar, Brooklyn Law School
- 1988-1990: Dean's List, Brooklyn Law School
- 1986-1988: American Cancer Society Postdoctoral Fellow, Sloan Kettering Institute



- 1984: Student Research Award, Delta Omega Honorary Public Health Society
- 1979-1986: Predoctoral Training Grants: Predoctoral trainee, NIH (1979-1982); Albert Szent-Gyorgyi Foundation (1982-1986)
- May 1979: Graduated with General Honors and Honors in Chemistry from Goucher College  
Louise Kelly Award in Chemistry, Goucher College
- 1979: Undergraduate Research Program (January-May) and Summer Graduate Student Program (June-August) at Argonne National Laboratory, Argonne, Illinois

### **FACULTY APPOINTMENTS**

Angel Financing: Navigating the Legal & Business Issues, Citybar Center for CLE, The Association of the Bar of the City of New York, November 28, 2000

Organizer and instructor at National Association of Patent Practitioner's 2000 Short Course on Nuts and Bolts of Patent Prosecution, July 2000

Instructor, Sixth, Eighth, Ninth and Tenth Annual Patent Prosecution Workshops: Advanced Claim Drafting and Amendment Writing (1996, 1998, 1999, 2000)

Law Seminars International: Biotechnology Key Legal & Business Issues, November 18-19, 1999, Seattle Washington

### **ADDITIONAL SKILLS**

Computer literate, LEXIS, WESTLAW, DIALOG, Internet User

### **MEMBERSHIPS:**

National Association of Patent Practitioners: Member, Board of Directors and Chairperson of Education Committee  
American Intellectual Property Law Association  
Eastern New York Intellectual Property Law Association  
American Bar Association, Intellectual Property Section  
Association of the Bar of the City of New York  
Association for Women in Science  
Association of University Transfer Managers  
Westchester Women's Bar Association  
International Intellectual Property Society

## ORAL PRESENTATIONS

"Alternative Career Opportunities in Intellectual Property Law", New York Biotechnology Association, Women in Bioesciences Section Meeting, June 2000

"Why Deposit Biological Materials?" New York, New Jersey, Connecticut, and Pennsylvania Joint Seminar on Developments in Patent Law, April 2000

"Inventorship", National Association of Patent Practitioners meeting, July 1999  
Panel chair, "Interactions Between In-House and Law Firm Patent Counsel to Develop Intellectual Property Strategy", BIO '98, June 1998

"What to Claim in Biotechnology Patent Applications", National Association of Patent Practitioners meeting, October 1997

"*In re Deuel*, Obviousness Standard for Biotechnology", BIO '96, June 1996

## PUBLICATIONS:

### Patents:

Have participated in the preparation and/or prosecution of over 150 patents. Representative patents are listed below:

U.S. Patent No. 6,060,305, "Non-toxic, non-toxigenic, non-pathogenic *Fusarium* expression system"

U.S. Patent No. 5,919,697, "Color Clarification Methods"

U.S. Patent No. 5,843,753, "Metalloprotease having increased activity"

U.S. Patent No. 5,770,371, "Modification of cryptic splice sites in heterologous genes expressed in fungi"

U.S. Patent No. 5,726,202, "Benign prostatic hypertrophy"

U.S. Patent No. 5,707,798, "Identification of ligands by selective amplification of cells transfected with receptors"

U.S. Patent No. 5,602,032, "*Bacillus thuringiensis* mutants which produce high yields of crystal delta-endotoxin"

U.S. Patent No. 5,580,560, "Modified factor VII/VIIa"

U.S. Patent No. 5,525,193, "Use of monocomponent cellulase for removing inks, coatings, and toners from printed paper"

U.S. Patent No. 5,354,760, "Crystalline Tiagabine monohydrate, its preparation and use"

### **Legal-related Publications:**

- Agris, C.H. (2000) "Biotechnology Applications: Depositing Biological Materials" Intellectual Property Today 7:12-13
- Agris, C.H. (1999) "Patenting Plants: What to Claim", Nature BioTechnology 17:717-718
- Agris, C.H. (1999) "Intellectual property protection for plants", Nature BioTechnology 17:197-198
- Agris, C.H. (1998) "Patenting Protein Sequences", Nature BioTechnology 16:1075
- Agris, C.H. (1998) "Patenting DNA Sequences", Nature BioTechnology 16:877
- Agris, C.H. (1998) "International Patent Filing", Nature BioTechnology 16:479-480
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Miller, P.S.; Reddy, M.P.; Murakami, A.; Blake, K.R.; Lin, S.B. and Agris, C.H. (1986) Solid-phase syntheses of oligodeoxyribonucleoside methylphosphonates Biochemistry 25:5092-5097

Miller, P.S.; Agris, C.H.; Aurelian, L.; Blake, K.R.; Lin, S.B.; Murakami, A.; Reddy, M.P.; Spitz, S.A. and Ts'o, O.P. (1985) Control of ribonucleic acid function by oligonucleoside methylphosphonates, Biochimie 67: 769-776

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Miller, P.S., Agris, C.H., Blake, K.R., Murakami, A., Spitz, S.A., Reddy, P.M. and Ts'o, P.O.P. (1983) Nonionic oligonucleotide analogs as new tools for studies on the structure and function of nucleic acids inside living cells. In Nucleic Acids: The Vectors of Life, B. Pullman and J. Jortner, ed. (D. Reidel Publishing Co., Boston), pp. 521-535

Effects of Chemically Synthesized Oligodeoxyribonucleoside  
Methylphosphonates on Vesicular Stomatitis Virus  
Protein Synthesis and Infection

by

Cheryl Heather Agris

A dissertation submitted to the Johns Hopkins University  
in conformity with the requirements for the degree of  
Doctor of Philosophy

Baltimore, MD

1986

## II.

## Background

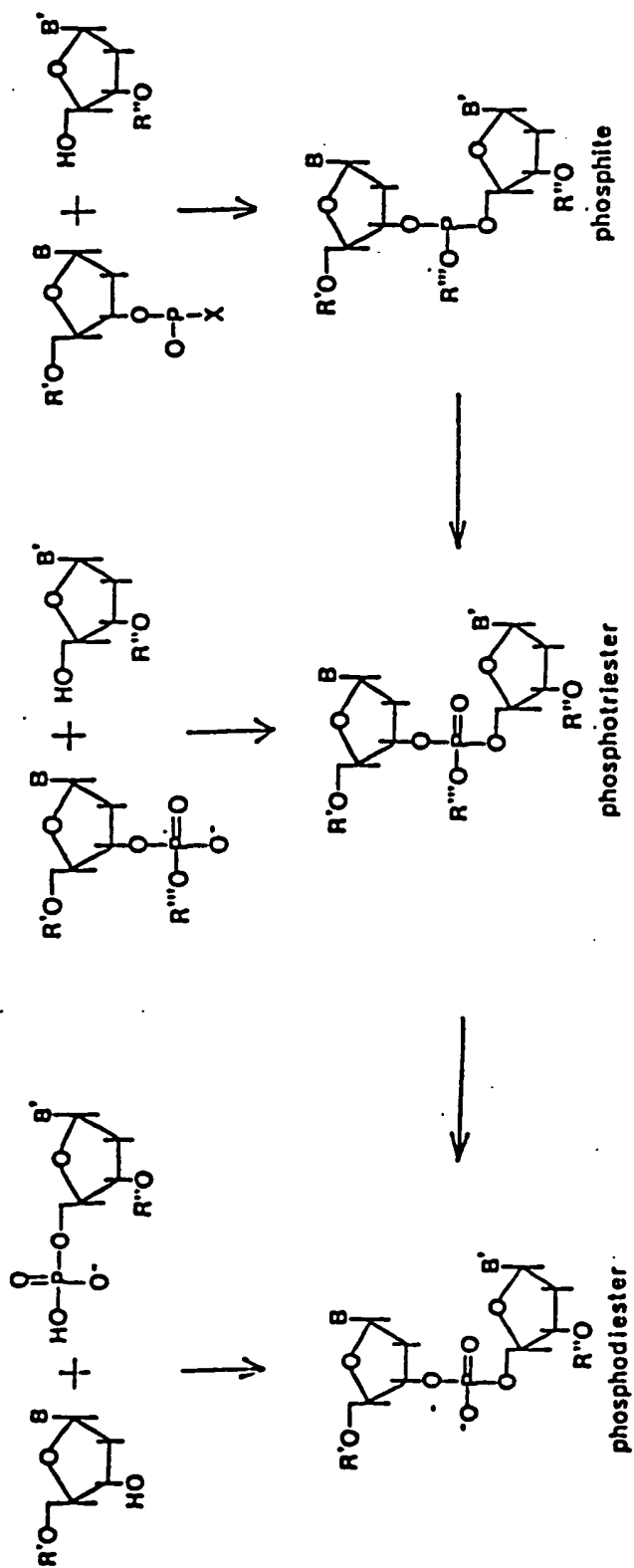
A. Synthesis of oligodeoxyribonucleotides and  
oligodeoxyribonucleoside methylphosphonates

## 1. Synthesis of oligodeoxyribonucleotides

The chemical synthesis of oligodeoxyribonucleotides involves the formation of phosphodiester internucleotide linkages. It has been accomplished by the activation of phosphomonoesters (phosphodiester method) (Jacob and Khorana, 1964), phosphodiesters (phosphotriester method) (Letsinger and Ogilvie, 1969), and phosphites (phosphite method) (Letsinger and Lunsford, 1976) (see figure 1). The reaction between a nucleotide and nucleoside to form an internucleotide linkage is a condensation reaction. All functional groups except for those involved in the condensation reaction must be blocked with protecting groups to prevent the formation of side products. These groups are removed at the conclusion of the condensation reaction.

The 5'-hydroxyl group is commonly blocked by one of a family of ether groups which are increasingly labile to acid in the order trityl < monomethoxytrityl < dimethoxytrityl (Schaller et al., 1963). The structures of these compounds are shown in figure 2. Since the amino group of the bases are reactive, they are protected with a benzoyl group in the case of adenine and cytidine (Schaller et al., 1963) and with an isobutyryl moiety in the case of guanine (Agarwal et al.,

Figure 1. Phosphodiester, phosphotriester, and phosphite methods for the preparation of dideoxynucleotides.



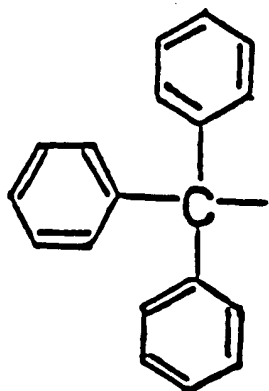
R' = trityl, monomethoxytrityl, dimethoxytrityl  
(see fig. 2 for structures)

R'' = -OCH<sub>3</sub>, -Si(CH<sub>3</sub>)<sub>2</sub>-C(CH<sub>3</sub>)<sub>3</sub>, -OC<sub>6</sub>H<sub>5</sub>

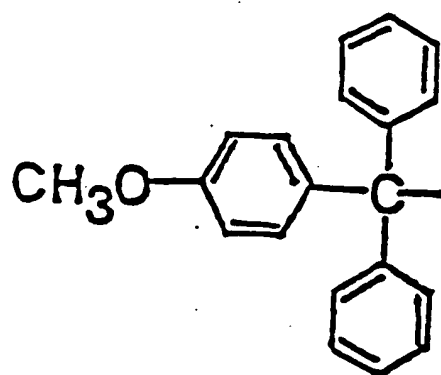
R''' = -CH<sub>2</sub>CH<sub>2</sub>CN, -pC<sub>6</sub>H<sub>4</sub>, -oC<sub>6</sub>H<sub>4</sub>, -CH<sub>3</sub>

X = -Cl, -N(CH<sub>3</sub>)<sub>2</sub>

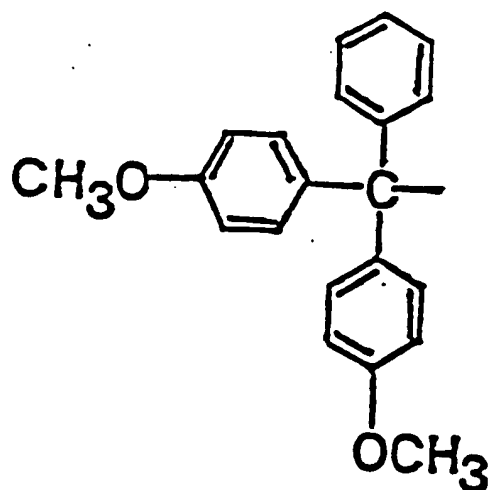




trityl



monomethoxytrityl



dimethoxytrityl

Figure 2. 5'-protecting groups

1972) (see figure 3). Both the isobutyryl and benzoyl groups are base labile. A number of adducts have been used to block the 3'-hydroxyl group, e.g. acetyl (OAc) (Agarwal et al., 1972) or t-butyldimethylsilyl (OTBS) adducts (Ogilvie, 1973). Since the acetyl group is base labile, the base protecting groups may be partially removed as well. This may not be desirable if a polynucleotide is being synthesized. The t-butyldimethylsilyl group can be removed by fluoride treatment.

Three groups are most commonly used to protect the phosphate linkage when the phosphotriester or phosphite approaches are used. No protecting group was used in the phosphodiester method. The  $\beta$ -cyanoethyl group has been used in the phosphotriester (Letsinger et al., 1969) and phosphite methods (Sinha et al., 1984). This group is removed by  $\beta$ -elimination using mild alkaline treatments. The o- and p-chlorophenyl derivatives were developed in connection with the phosphotriester method (Katagiri et al., 1975).

These groups can be selectively removed by tetramethylguanidium pyridine aldoximate (Reese et al., 1978), a mild nucleophile. The methyl group has been developed for use in the phosphite method and is removed by thiophenol followed by treatment with concentrated ammonium hydroxide (Matteucci and Caruthers, 1981). The o- and p-chlorophenyl and methyl protecting groups are not interchangeable between synthetic methods since it is virtually impossible to make chlorophenyl phosphitylating

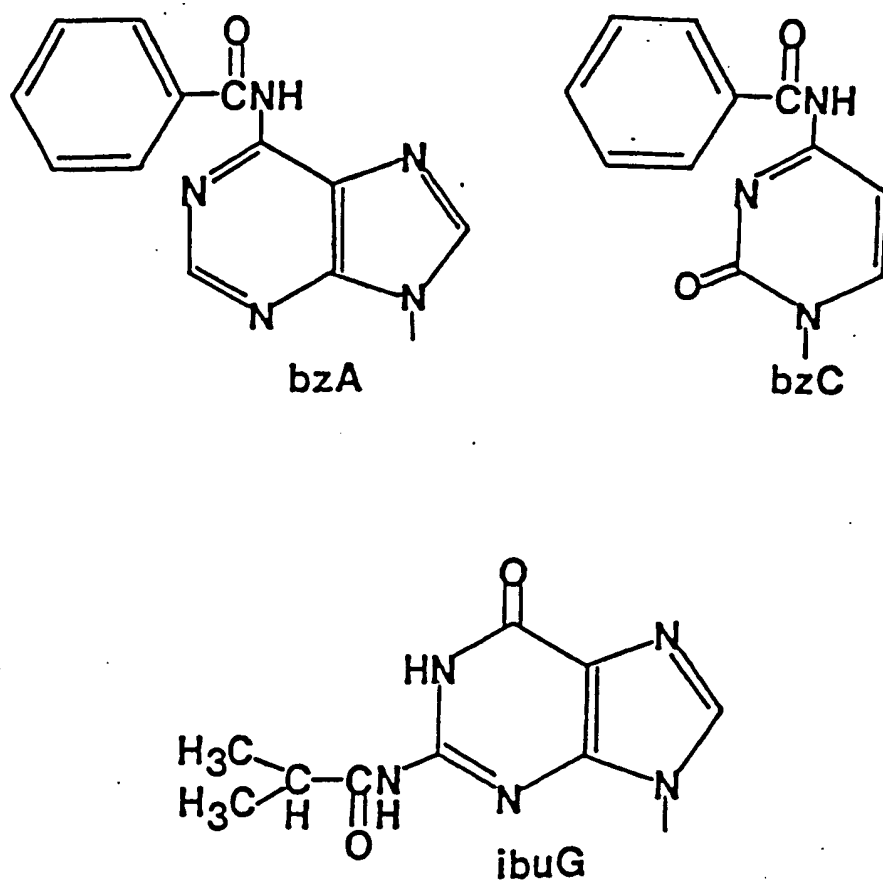


Figure 3. Base protecting groups

agents and methyl substituted phosphorylating agents are not very reactive (Gait, 1984).

As shown in figure 1, the phosphodiester approach involves direct condensation of oligonucleotides bearing a 5'-phosphomonoester group with oligonucleotides possessing a free terminal 3'-hydroxyl group. Originally, the phosphomonoester group was activated with dicyclohexylcarbodiimide (Jacob and Khorana, 1964). Faster activation was found to be achieved with such arenesulfonyl chlorides as mesitylenesulfonyl chloride or triisopropylbenzenesulfonyl chloride (Lohrmann and Khorana, 1965). The major drawback to this approach is that the phosphodiester linkages in the internucleotide linkages are also activated by these condensing agents resulting in the formation of side products with 3'-3' internucleotide linkages.

In the phosphotriester method, all hydroxyl groups are protected, thereby eliminating the 3'-3' side product (Katagiri et al., 1975). However sulfonated side products were still present when arenesulfonyl chlorides were used as condensing agents. In an effort to circumvent this problem and to decrease the reaction times, more active derivatives were introduced. These included arenesulfonyl triazolides (Katagiri et al., 1975), arenesulfonyl tetrazolides (Stawinski et al., 1977), and later mesitylenesulfonyl-3-nitrotriazole (MSNT) (Reese et al., 1978).

The third approach or phosphite method involves reacting

a protected nucleoside with a bifunctional phosphitylating agent such as methoxydichlorophosphine (Letsinger and Lunsford, 1976). The resulting nucleoside 3'-phosphomonochloridite is then reacted with a second protected nucleoside resulting in the formation of a dinucleoside phosphite. This product is converted to a phosphate by oxidation with iodine. Condensation reactions in which the phosphite method is used are completed faster than those using phosphotriester chemistry. The main disadvantage to this method is that due to its instability, the preparation of the phosphomonochloridate must be carried out at  $-78^{\circ}\text{C}$ . There is also considerable formation of 3'-3' dimer. In an effort to overcome these disadvantages, phosphoramidite derivatives of the nucleoside phosphomonochloridite were prepared (Beaucage and Caruthers, 1981). These intermediates have been found to be stable and can be activated in the presence of tetrazole to form the internucleotide bond.

Oligodeoxyribonucleotides may be synthesized in solution or on a solid polymer support. The solution method has the following advantages (Ohtsuka et al., 1982): 1) Relatively large quantities of materials (10-100 mg) can be synthesized. 2) The ratio of 3' to 5' components used in the condensation reactions are usually in the range of 1.5 to 2.0 equivalents. Thus, large excesses of reagents are not required. The major disadvantage of the solution method is that protected oligonucleotides must be purified after each condensation

step. This purification is usually carried out by silica gel column chromatography (Miller et al., 1980).

Oligodeoxyribonucleotides have also been synthesized on a solid polymer support using both the phosphotriester (Ito et al., 1982) and phosphite approaches (Matteucci and Caruthers, 1981). In this method, the growing oligomer chain is attached to a solid matrix via a linker arm. The growing oligonucleotide chain is therefore purified after each condensation step by a simple filtration and washing procedure. Examples of solid support materials used in oligonucleotide synthesis include polydimethylacrylamide (Gait et al., 1980), polyacrylomorpholide (Miyoshi et al., 1980), silica gel (Matteucci and Caruthers, 1981), polystyrene (Ito et al., 1982), and controlled pore glass (Sproat et al., 1983). Although the method is quite rapid, it appears that rather large excesses (5-20 fold) of incoming nucleotides are required for reactions to go in high yield. It should also be noted that the scale of the synthesis can be reduced since the intermediates need not be isolated after each condensation step. Recently, the operations involved in solid support synthesis have been automated by a number of companies, resulting in the marketing of synthesis machines.

After synthesis of deoxyribonucleotides in solution or on a solid support, all protecting groups are removed. The oligomer is then commonly purified by preparative High Pressure Liquid Chromatography (HPLC) on an ion exchange or

reversed phase column (Miller et al., 1980). Recently, however, a new method has been devised for purifying oligomers by gel electrophoresis (Lo et al., 1984). The final oligomer can then be characterized by HPLC on a reversed phase column and by sequencing using the Maxam-Gilbert procedure (Maxam and Gilbert, 1980).

## 2. Synthesis of oligodeoxyribonucleoside methylphosphonates

Oligodeoxyribonucleoside methylphosphonates have been synthesized by a number of groups both in solution and on a solid polymer support. Initially, Miller et al. (1979) used mesitylenesulfonyl tetrazolide as a condensing agent in the reaction between a 5'-protected nucleoside 3'-methylphosphonate and a 3'-O-acetylated nucleoside. The 5'-protected nucleoside 3'-methylphosphonate is prepared by esterification of protected nucleosides with methylphosphonic acid in the presence of dicyclohexylcarbodiimide. This procedure is analogous to that used for the preparation of oligonucleoside phosphotriesters (Letsinger and Ogilvie, 1969). Yields were approximately 40-55%.

Agarwal and Riftina (1979) reported the use of methylphosphonic dichloride in combination with benzenesulfonyl tetrazolide as a phosphorylating and condensing agent. In this approach, methylphosphonic dichloride was reacted with 5'-protected nucleoside to prepare 5'-protected nucleoside methylphosphonic chlorides. The intermediate is further activated by benzenesulfonyl

tetrazolide, which is added in the presence of the second nucleoside. They found that when methylphosphonic dichloride was used alone, low yields (12%) were obtained and long reaction times were required.

Our laboratory though has found that with modified reaction conditions that methylphosphonic dichloride can act as a bifunctional phosphorylating/condensing agent to prepare nucleoside 3'-methylphosphonic chlorides and protected di- and trinucleoside methylphosphonates (Miller et al., 1983a). This procedure represented a considerable improvement in time and yield obtained in the preparation of reaction intermediates and di- and trinucleoside methylphosphonates over the method previously used (Miller et al., 1979).

Our laboratory has also synthesized oligonucleoside methylphosphonates on silica gel and 1% divinylbenzene crosslinked polystyrene solid supports. 5'-protected 3'-methylphosphonic chlorides were used as synthetic intermediates when preparing these analogs on a silica gel support (Miller et al., 1983a). Although yields were approximately 70% per step when oligothymidylates were prepared on a silica gel support using this procedure, reactions were less efficient for other nucleosides especially 5'-protected G. Oligomers up to 12 nucleoside units have been prepared in which the triethylammonium salt of 5'-protected nucleoside 3'-methylphosphonic acid is reacted with a nucleoside or oligonucleoside methylphosphonate attached to a



1%-divinylbenzene crosslinked polystyrene solid support with MSNT as the condensing agent (Miller et al., 1983b). Yields have averaged 83% per step. The use of MSNT however can potentially lead to formation of side products resulting from sulfonylation of the nucleoside or 5'-hydroxyl groups. These side products lower the overall yield and complicate purification of the oligomer. In an effort to avoid side reactions, we have recently tested the effectiveness of 5'-protected 3'-methylphosphonic imidazolides as reaction intermediates (Miller et al., 1985b). These reagents contain a leaving group which can be activated just prior to the condensation reaction. This method will be discussed in further detail in later sections of this thesis.

Oligodeoxyribonucleoside methylphosphonates have also been synthesized in solution and on solid supports using methods analogous to the phosphite procedure. One approach involves the use of dichloromethylphosphine as a bifunctional phosphinylating and condensing agent (Engels and Jager, 1982). In this method, 5'-protected 3'-methylphosphine chlorides were used as reactive intermediates in the preparation of oligodeoxyribonucleoside methylphosphonates both in solution (Engels and Jager, 1982) and on a controlled pore glass solid support (Sinha et al., 1983). Dideoxynucleoside methylphosphonates have also been synthesized in solution using nucleoside methylphosphonamidites as reaction intermediates (Jager and Engels, 1984). These compounds have

Dean L. Engelhardt, *et al.*  
Serial No.: 08/479,997  
Filed: June 7, 1995  
Page 2 (Amendment Under 37 C.F.R. § 1.116 - November 20, 1998)

PREVIOUSLY PENDING CLAIMS 454-575  
EXHIBIT 3 TO DECLARATION OF DR. CHERYL H. AGRIS,  
ATTORNEY AT LAW (IN SUPPORT OF THE WRITTEN  
DESCRIPTION, ENABLEMENT & NON-OBVIOUSNESS OF  
THE INVENTION CLAIMED IN U.S. PATENT APPLICATION  
SERIAL NO.  
08/479,997

**AMEND THE ABOVE-IDENTIFIED APPLICATION AS FOLLOWS:**

**In The Specification:**

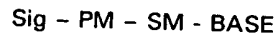
Page 53, line 22, after "ribonucleotide. The" and before "vicinal"  
change "3', 4'" to -- 3', 2' -- .

**In The Claims:**

Cancel claims 310-372 and 405-453.

Add new claims 454-573 as follows:

-- 454. (NEW) An oligo- or polydeoxyribonucleotide comprising at least one nucleotide having the formula

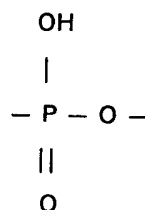


wherein PM is a phosphate moiety, SM is a sugar moiety and BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, said PM being attached to SM, said BASE being attached to SM, and Sig being covalently attached to PM directly or via a chemical linkage, said Sig being a moiety capable of non-radioactive detection when attached to PM or when said nucleotide is incorporated into said oligo- or polydeoxyribonucleotide. --

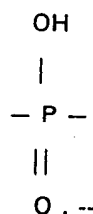
-- 455. (NEW) The oligo- or polydeoxyribonucleotide of claim 454, wherein said Sig is or renders the nucleotide self-signaling or self-indicating or self-detecting. --

-- 456. (NEW) The oligo- or polydeoxyribonucleotide of claim 454, wherein said Sig moiety comprises at least three carbon atoms. --

-- 457. (NEW) The oligo- or polydeoxyribonucleotide of claim 454, wherein said covalent attachment is selected from the group consisting of



and

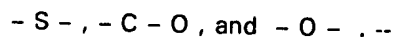
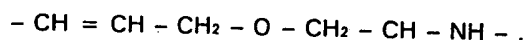
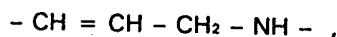
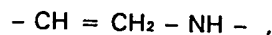


-- 458. (NEW) The oligo- or polydeoxyribonucleotide of claim 454, wherein said chemical linkage does not interfere substantially with the characteristic ability of Sig to form a detectable signal. --

-- 459. (NEW) The oligo- or polydeoxyribonucleotide of claim 454, wherein said chemical linkage comprises a member selected from the group consisting of an olefinic bond at the alpha-position relative to the point of attachment to the nucleotide, a  $-\text{CH}_2\text{NH}-$  moiety, or both. --

-- 460. (NEW) The oligo- or polydeoxyribonucleotide of claim 454, wherein said chemical linkage comprises an allylamine group. --

-- 461. (NEW) The oligo- or polydeoxyribonucleotide of claim 454, wherein said chemical linkage comprises or includes an olefinic bond at the delta-position relative to the point of attachment to the nucleotide, or any of the moieties:



-- 462. (NEW) The oligo- or polydeoxyribonucleotide of claim 454, wherein said chemical linkage of Sig includes a glycosidic linkage moiety. --

<sup>AMENDED</sup>  
463. (Re-written) The oligo- or polydeoxyribonucleotide of claim 454, wherein said PM is a monophosphate, a diphosphate or a triphosphate and said Sig moiety is covalently attached to said PM through a phosphorus atom or a phosphate oxygen.

-- 464. (NEW) The oligo- or polydeoxyribonucleotide of claim 454, wherein Sig comprises a component selected from the group consisting of biotin, iminobiotin, an electron dense component, a magnetic component, an enzyme or an enzyme component, a hormone or a hormone component, a metal-containing component, a fluorescent component, a chemiluminescent component, an antigen, a hapten and an antibody or an antibody component, or a combination of any of the foregoing. --

-- 465. (NEW) The oligo- or polydeoxyribonucleotide of claim 464, wherein said electron dense component comprises ferritin. --

- 466. (NEW) The oligo- or polydeoxyribonucleotide of claim 454, wherein Sig is complexed with a binding protein therefor, and said binding protein is conjugated to ferritin. --
- 467. (NEW) The oligo- or polydeoxyribonucleotide of claim 464, wherein said magnetic component comprises magnetic oxide. --
- 468. (NEW) The oligo- or polydeoxyribonucleotide of claim 467, wherein said magnetic oxide comprises ferric oxide. --
- 469. (NEW) The oligo- or polydeoxyribonucleotide of claim 464, wherein said enzyme or enzyme component is selected from the group consisting of alkaline phosphatase, acid phosphatase,  $\beta$ -galactosidase, ribonuclease, glucose oxidase and peroxidase. --
- 470. (NEW) The oligo- or polydeoxyribonucleotide of claim 464, wherein said metal-containing component is catalytic. --
- 471. (NEW) The oligo- or polydeoxyribonucleotide of claim 464, wherein said fluorescent component comprises a member selected from the group consisting of fluorescein, rhodamine and dansyl. --
- 472. (NEW) The oligo- or polydeoxyribonucleotide of claim 464, wherein Sig is selected from the group consisting of an antigen or hapten capable of complexing with an antibody or antibody component specific thereto, and an antibody or antibody component capable of complexing with an antigen or hapten. --
- 473. (NEW) The oligo- or polydeoxyribonucleotide of claim 454, wherein said oligo- or polydeoxyribonucleotide is terminally ligated or attached to a polypeptide. --
- 474. (NEW) A composition comprising the oligo- or polydeoxyribonucleotide of claim 454, a polypeptide capable of forming a complex with Sig and a moiety which can be detected when such complex is formed. --

Dean L. Engelhardt, *et al.*

Serial No.: 08/479,997

Filed: June 7, 1995

Page 6 (Amendment Under 37 C.F.R. § 1.116 - November 20, 1998)

-- 475. (NEW) The composition of claim 474, wherein said polypeptide comprises polylysine. --

-- 476. (NEW) The composition of claim 474, wherein said polypeptide is selected from the group consisting of avidin, streptavidin and anti-Sig immunoglobulin. --

--477. (NEW) The composition of claim 474, wherein Sig is a ligand and said polypeptide is an antibody thereto. --

-- 478. (NEW) The oligo- or polydeoxyribonucleotide of claim 454, wherein said Sig moiety is attached to a terminal nucleotide in said oligo- or polydeoxyribonucleotide. --

AMENDED

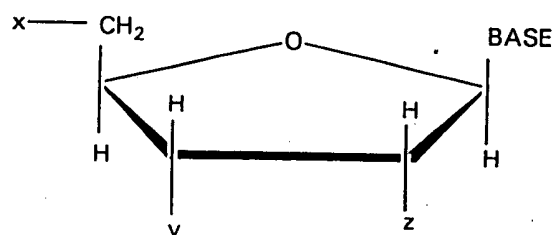
479. (~~Re-written~~) The oligo- or polydeoxyribonucleotide of claim 478, wherein the sugar moiety of said terminal nucleotide has a hydrogen atom at the 2' position thereof.

AMENDED

480. (~~Re-written~~) The oligo- or polydeoxyribonucleotide of claim 478, wherein the sugar moiety of said terminal nucleotide has hydrogen atoms at each of the 2' and 3' positions thereof.

-- 481. (NEW) The oligo- or polydeoxyribonucleotide of claim 454, comprising at least one ribonucleotide. --

-- 482. (NEW) An oligo- or polydeoxribonucleotide comprising at least one nucleotide having the structural formula:



wherein BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, and wherein BASE is attached to the 1' position of the pentose ring from the N1 position when BASE is a pyrimidine or from the N9 position when BASE is a purine or a deazapurine;

wherein x is selected from the group consisting of H-, HO-, a mono-phosphate, a di-phosphate and a tri-phosphate;

wherein y is selected from the group consisting of H-, HO-, a mono-phosphate, a di-phosphate and a tri-phosphate;

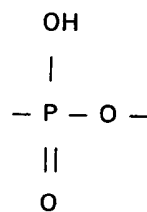
wherein z is H-; and

wherein Sig is covalently attached to x, y or z directly or through a chemical linkage, said Sig being a moiety capable of non-radioactive detection when so attached to x, y or z. --

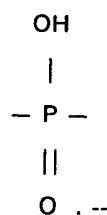
-- 483. (NEW) The oligo- or polydeoxyribonucleotide of claim 482, wherein said Sig is or renders the nucleotide or the oligo- or polynucleotide self-signaling or self-indicating or self-detecting. --

-- 484. (NEW) The oligo- or polydeoxyribonucleotide of claim 482, wherein said Sig moiety comprises at least three carbon atoms. --

-- 485. (NEW) The oligo- or polydeoxyribonucleotide of claim 482, wherein said covalent attachment is selected from the group consisting of



and



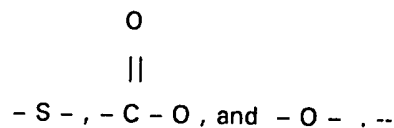
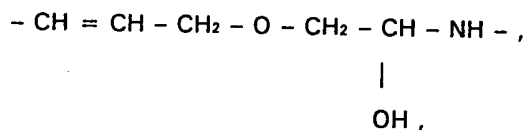
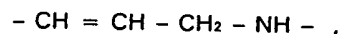
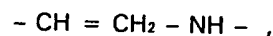
-- 486. (NEW) The oligo- or polydeoxyribonucleotide of claim 482, wherein said chemical linkage does not interfere substantially with the characteristic ability of Sig to form a detectable signal. --

-- 487. (NEW) The oligo- or polydeoxyribonucleotide of claim 482, wherein said chemical linkage comprises a member selected from the group consisting of an olefinic bond at the alpha-position relative to the point of attachment to the nucleotide, a  $-\text{CH}_2\text{NH}-$  moiety, or both. --

-- 488. (NEW) The oligo- or polydeoxyribonucleotide of claim 482, wherein said chemical linkage comprises an allylamine group. --



-- 489. (NEW) The oligo- or polydeoxyribonucleotide of claim 482, wherein said chemical linkage comprises or includes an olefinic bond at the delta-position relative to x, y or z, or any of the moieties:



-- 490. (NEW) The oligo- or polydeoxyribonucleotide of claim 482, wherein said chemical linkage of Sig includes a glycosidic linkage moiety. --

<sup>AMENDED</sup>  
491. (~~REVISION~~) The oligo- or polydeoxyribonucleotide of claim 482, wherein said x and y each comprise a member selected from the group consisting of a monophosphate, a diphosphate and a triphosphate and said Sig moiety is covalently attached to either or both of said x and y through a phosphorus atom or a phosphate oxygen.

-- 492. (NEW) The oligo- or polydeoxyribonucleotide of claim 482, wherein Sig comprises a component selected from the group consisting of biotin, iminobiotin, an electron dense component, a magnetic component, an enzyme or an enzyme component, a hormone or a hormone component, a metal-containing component, a fluorescent component, a chemiluminescent component, an antigen, a hapten and an antibody or an antibody component, or a combination of any of the foregoing. --

-- 493. (NEW) The oligo- or polydeoxyribonucleotide of claim 492, wherein said electron dense component comprises ferritin. --

- 494. (NEW) The oligo- or polydeoxyribonucleotide of claim 482, wherein Sig is complexed with a binding protein therefor, and said binding protein is conjugated to ferritin. --
- 495. (NEW) The oligo- or polydeoxyribonucleotide of claim 492, wherein said magnetic component comprises magnetic oxide. --
- 496. (NEW) The oligo- or polydeoxyribonucleotide of claim 495, wherein said magnetic oxide comprises ferric oxide. --
- 497. (NEW) The oligo- or polydeoxyribonucleotide of claim 492, wherein said enzyme or enzyme component is selected from the group consisting of alkaline phosphatase, acid phosphatase,  $\beta$ -galactosidase, ribonuclease, glucose oxidase and peroxidase. --
- 498. (NEW) The oligo- or polydeoxyribonucleotide of claim 492, wherein said metal-containing component is catalytic. --
- 499. (NEW) The oligo- or polydeoxyribonucleotide of claim 492, wherein said fluorescent component comprises a member selected from the group consisting of fluorescein, rhodamine and dansyl. --
- 500. (NEW) The oligo- or polydeoxyribonucleotide of claim 492, wherein Sig is selected from the group consisting of an antigen or hapten capable of complexing with an antibody or antibody component specific thereto, and an antibody or antibody component capable of complexing with an antigen or hapten. --
- 501. (NEW) The oligo- or polydeoxyribonucleotide of claim 482, wherein said oligo- or polydeoxyribonucleotide is terminally ligated or attached to a polypeptide. --
- 502. (NEW) A composition comprising the oligo- or polydeoxyribonucleotide of claim 482, a polypeptide capable of forming a complex with Sig and a moiety which can be detected when such complex is formed. --

Dean L. Engelhardt, *et al.*

Serial No.: 08/479,997

Filed: June 7, 1995

Page 11 (Amendment Under 37 C.F.R. § 1.116 - November 20, 1998)

-- 503. (NEW) The composition of claim 500, wherein said polypeptide comprises polylysine. --

-- 504. (NEW) The composition of claim 502, wherein said polypeptide is selected from the group consisting of avidin, streptavidin and anti-Sig immunoglobulin. --

-- 505. (NEW) The composition of claim 502, wherein Sig is a ligand and said polypeptide is an antibody thereto. --

-- 506. (NEW) The oligo- or polydeoxyribonucleotide of claim 482, wherein said Sig moiety is attached to a terminal nucleotide in said oligo- or polydeoxyribonucleotide. --

507. <sup>AMENDED</sup> (Rewritten) The oligo- or polydeoxyribonucleotide of claim 506, wherein z of said terminal nucleotide comprises a hydrogen atom at the 2' position thereof.

508. <sup>AMENDED</sup> (Rewritten) The oligo- or polydeoxyribonucleotide of claim 506, wherein both y and z of said terminal nucleotide comprise a hydrogen atom at each of the 3' and 2' positions thereof, respectively.

-- 509. (NEW) The oligo- or polydeoxyribonucleotide of claim 482, comprising at least one ribonucleotide. --

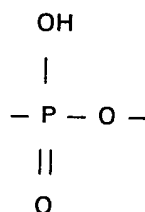
The diagram illustrates a branched polysaccharide structure. It features three pyranose rings in chair conformations, linked by 1,6-glycosidic bonds. The leftmost unit is enclosed in brackets with a subscript 'm'. It has a phosphate group attached to its C6 via a dashed bond, and a substituent B' at C2. The middle unit is linked to the first unit at its C1 and has a substituent B...A at its C2. The rightmost unit is enclosed in brackets with a subscript 'n' and is linked to the middle unit at its C1. It has a phosphate group attached to its C6 via a dashed bond and a substituent B'' at its C2. All phosphate groups are shown with a double bond to an oxygen and two hydroxyl groups.

**Enz-5(D6)(C2)**

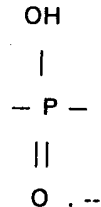
-- 512. (NEW) The oligo- or polyribonucleotide of claim 511, wherein said Sig is or renders the nucleotide self-signaling or self-indicating or self-detecting. --

-- 513. (NEW) The oligo- or polyribonucleotide of claim 511, wherein said Sig moiety comprises at least three carbon atoms. --

-- 514. (NEW) The oligo- or polyribonucleotide of claim 511, wherein said covalent attachment is selected from the group consisting of



and



-- 515. (NEW) The oligo- or polyribonucleotide of claim 511, wherein said chemical linkage does not interfere substantially with the characteristic ability of Sig to form a detectable signal. --

-- 516. (NEW) The oligo- or polyribonucleotide of claim 511, wherein said chemical linkage comprises a member selected from the group consisting of an olefinic bond at the alpha-position relative to the point of attachment to the nucleotide, a  $-\text{CH}_2\text{NH}-$  moiety, or both. --

-- 517. (NEW) The oligo- or polyribonucleotide of claim 511, wherein said chemical linkage comprises an allylamine group. --

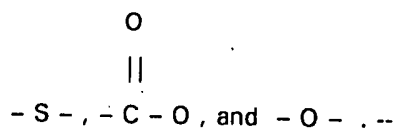
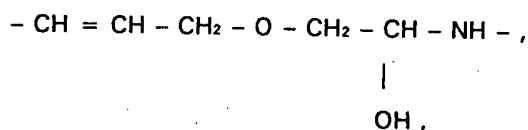
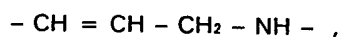
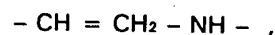
Dean L. Engelhardt, et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 14 (Amendment Under 37 C.F.R. § 1.116 - November 20, 1998)

-- 518. (NEW) The oligo- or polyribonucleotide of claim 511, wherein said chemical linkage comprises or includes an olefinic bond at the delta-position relative to the point of attachment to the nucleotide, or any of the moieties:



-- 519. (NEW) The oligo- or polyribonucleotide of claim 511, wherein said chemical linkage of Sig includes a glycosidic linkage moiety. --

~~520. (Amended)~~ <sup>AMENDED</sup> The oligo- or polyribonucleotide of claim 511, wherein said PM is a monophosphate, a diphosphate or a triphosphate and said Sig moiety is covalently attached to said PM through a phosphorus atom or a phosphate oxygen.

-- 521. (NEW) The oligo- or polyribonucleotide of claim 511, wherein Sig comprises a component selected from the group consisting of biotin, iminobiotin, an electron dense component, a magnetic component, an enzyme or an enzyme component, a hormone or a hormone component, a metal-containing component, a fluorescent component, a chemiluminescent component, an antigen, a hapten and an antibody or an antibody component, or a combination of any of the foregoing. --

-- 522. (NEW) The oligo- or polyribonucleotide of claim 521, wherein said electron dense component comprises ferritin. --

-- 523. (NEW) The oligo- or polyribonucleotide of claim 511, wherein Sig is complexed with a binding protein therefor, and said binding protein is conjugated to ferritin. --

-- 524. (NEW) The oligo- or polyribonucleotide of claim 521, wherein said magnetic component comprises a magnetic oxide. --

-- 525. (NEW) The oligo- or polyribonucleotide of claim 524, wherein said magnetic oxide comprises ferric oxide. --

-- 526. (NEW) The oligo- or polyribonucleotide of claim 521, wherein said enzyme or enzyme component is selected from the group consisting of alkaline phosphatase, acid phosphatase,  $\beta$ -galactosidase, ribonuclease, glucose oxidase and peroxidase. --

-- 527. (NEW) The oligo- or polyribonucleotide of claim 521, wherein said metal-containing component is catalytic. --

-- 528. (NEW) The oligo- or polyribonucleotide of claim 521, wherein said fluorescent component comprises a member selected from the group consisting of fluorescein, rhodamine and dansyl. --

-- 529. (NEW) The oligo- or polyribonucleotide of claim 521, wherein Sig is selected from the group consisting of an antigen or hapten capable of complexing with an antibody or antibody component specific thereto, and an antibody or antibody component capable of complexing with an antigen or hapten. --

-- 530. (NEW) The oligo- or polyribonucleotide of claim 511, wherein said oligo- or polyribonucleotide is terminally ligated or attached to a polypeptide. --

-- 531. (NEW) A composition comprising the oligo- or polyribonucleotide of claim 511, a polypeptide capable of forming a complex with Sig and a moiety which can be detected when such complex is formed. --

-- 532. (NEW) The composition of claim 531, wherein said polypeptide comprises polylysine. --

Enz-5(D6)(C2)

Dean L. Engelhardt, *et al.*

Serial No.: 08/479,997

Filed: June 7, 1995

Page 16 (Amendment Under 37 C.F.R. § 1.116 - November 20, 1998)

-- 533. (NEW) The composition of claim 531, wherein said polypeptide is selected from the group consisting of avidin, streptavidin and anti-Sig immunoglobulin. --

-- 534. (NEW) The composition of claim 531, wherein Sig is a ligand and said polypeptide is an antibody thereto. --

-- 535. (NEW) The oligo- or polyribonucleotide of claim 511, wherein said Sig moiety is attached to a terminal ribonucleotide in said oligo- or polyribonucleotide. --

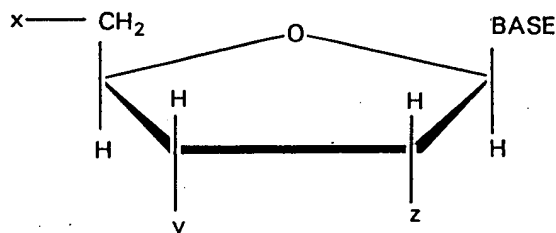
-- 536. (NEW) The oligo- or polyribonucleotide of claim 535, wherein the sugar moiety of said terminal ribonucleotide has a hydrogen atom at the 2' position thereof. --

-- 537. (NEW) The oligo- or polyribonucleotide of claim 535, wherein the sugar moiety of said terminal nucleotide has a hydrogen atom at each of the 2' and 3' positions thereof. --

-- 538. (NEW) The oligo- or polyribonucleotide of claim 511, comprising at least one deoxyribonucleotide. --



-- 539. (NEW) An oligo- or polyribonucleotide comprising at least one nucleotide having the structural formula:



wherein BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, and wherein BASE is attached to the 1' position of the pentose ring from the N1 position when BASE is a pyrimidine or from the N9 position when BASE is a purine or a deazapurine;

wherein x is selected from the group consisting of H-, HO-, a mono-phosphate, a di-phosphate and a tri-phosphate;

wherein y is selected from the group consisting of HO-, a mono-phosphate, a di-phosphate and a tri-phosphate;

wherein z is HO-; and

wherein Sig is covalently attached to x, y or z directly or through a chemical linkage, said Sig being a moiety capable of non-radioactive detection when so attached to x, y or z, provided that when Sig is attached through a chemical linkage to y of a terminal ribonucleotide, said chemical linkage is not a cleaved 3' terminal ribonucleotide previously attached to said oligo- or polyribonucleotide. --

-- 540. (NEW) The oligo- or polyribonucleotide of claim 539, wherein said Sig is or renders the nucleotide or the oligo- or polynucleotide self-signaling or self-indicating or self-detecting. --

-- 541. (NEW) The oligo- or polyribonucleotide of claim 539, wherein said Sig moiety comprises at least three carbon atoms. --

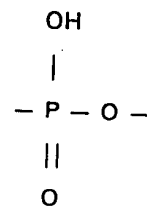
Dean L. Engelhardt, *et al.*

Serial No.: 08/479,997

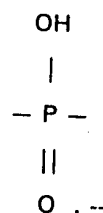
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Page 18 (Amendment Under 37 C.F.R. § 1.116 – November 20, 1998)

-- 542. (NEW) The oligo- or polyribonucleotide of claim 539, wherein said covalent attachment is selected from the group consisting of



and

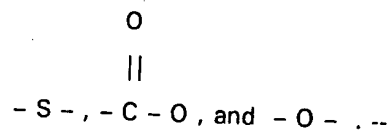
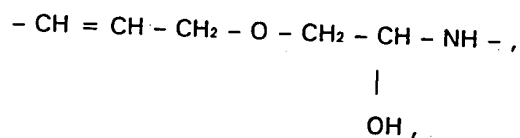
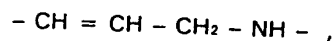
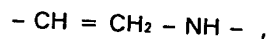


-- 543. (NEW) The oligo- or polyribonucleotide of claim 539, wherein said chemical linkage does not interfere substantially with the characteristic ability of Sig to form a detectable signal. --

-- 544. (NEW) The oligo- or polyribonucleotide of claim 539, wherein said chemical linkage comprises a member selected from the group consisting of an olefinic bond at the alpha-position relative to the point of attachment to the nucleotide, a  $-\text{CH}_2\text{NH}-$  moiety, or both. --

-- 545. (NEW) The oligo- or polyribonucleotide of claim 539, wherein said chemical linkage comprises an allylamine group. --

-- 546. (NEW) The oligo- or polyribonucleotide of claim 539, wherein said chemical linkage comprises or includes an olefinic bond at the delta-position relative to x, y or z, or any of the moieties:



-- 547. (NEW) The oligo- or polyribonucleotide of claim 539, wherein said chemical linkage of Sig includes a glycosidic linkage moiety. --

**AMENDED**

548. (~~Re-written~~) The oligo- or polyribonucleotide of claim 539, wherein said x and y each comprise a member selected from the group consisting of a monophosphate, a diphosphate and a triphosphate and said Sig moiety is covalently attached to either or both of said x and y through a phosphorus atom or a phosphate oxygen.

-- 549. (NEW) The oligo- or polyribonucleotide of claim 539, wherein Sig comprises a component selected from the group consisting of biotin, iminobiotin, an electron dense component, a magnetic component, an enzyme or an enzyme component, a hormone or a hormone component, a metal-containing component, a fluorescent component, a chemiluminescent component, an antigen, a hapten and an antibody or an antibody component, or a combination of any of the foregoing. --

-- 550. (NEW) The oligo- or polyribonucleotide of claim 549, wherein said electron dense component comprises ferritin. --

- 551. (NEW) The oligo- or polyribonucleotide of claim 539, wherein Sig is complexed with a binding protein therefor, and said binding protein is conjugated to ferritin. --
- 552. (NEW) The oligo- or polyribonucleotide of claim 549, wherein said magnetic component comprises magnetic oxide. --
- 553. (NEW) The oligo- or polyribonucleotide of claim 552, wherein said magnetic oxide comprises ferric oxide. --
- 554. (NEW) The oligo- or polyribonucleotide of claim 549, wherein said enzyme or enzyme component is selected from the group consisting of alkaline phosphatase, acid phosphatase,  $\beta$ -galactosidase, ribonuclease, glucose oxidase and peroxidase. --
- 555. (NEW) The oligo- or polyribonucleotide of claim 549, wherein said metal-containing component is catalytic. --
- 556. (NEW) The oligo- or polyribonucleotide of claim 549, wherein said fluorescent component comprises a member selected from the group consisting of fluorescein, rhodamine and dansyl. --
- 557. (NEW) The oligo- or polyribonucleotide of claim 549, wherein Sig is selected from the group consisting of an antigen or hapten capable of complexing with an antibody or antibody component specific thereto, and an antibody or antibody component capable of complexing with an antigen or hapten. --
- 558. (NEW) The oligo- or polyribonucleotide of claim 539, wherein said oligo- or polyribonucleotide is terminally ligated or attached to a polypeptide. --
- 559. (NEW) A composition comprising the oligo- or polyribonucleotide of claim 539, a polypeptide capable of forming a complex with Sig and a moiety which can be detected when such complex is formed. --
- 560. (NEW) The composition of claim 559, wherein said polypeptide comprises polylysine. --

-- 561. (NEW) The composition of claim 559, wherein said polypeptide is selected from the group consisting of avidin, streptavidin and anti-Sig immunoglobulin. --

-- 562. (NEW) The composition of claim 559, wherein Sig is a ligand and said polypeptide is an antibody thereto. --

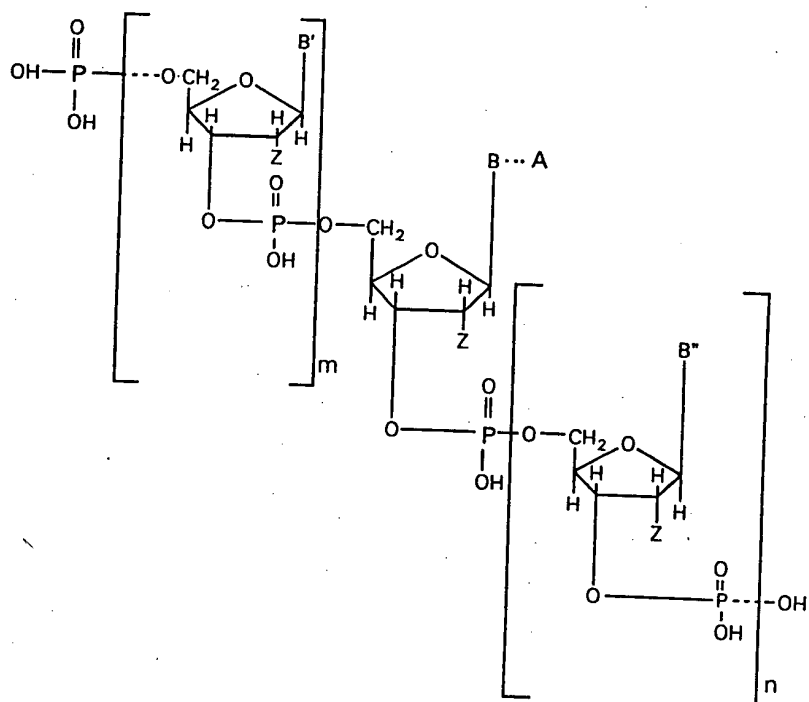
-- 563. (NEW) The oligo- or polyribonucleotide of claim 539, wherein said Sig moiety is attached to a terminal nucleotide in said oligo- or polyribonucleotide. --

~~564. (Revritten)~~ <sup>AMENDED</sup> The oligo- or polyribonucleotide of claim 563, wherein z of said terminal nucleotide comprises a hydrogen atom at the 2' position thereof.

~~565. (Revritten)~~ <sup>AMENDED</sup> The oligo- or polyribonucleotide of claim 563, wherein both y and z of said terminal nucleotide comprise a hydrogen atom at each of the 3' and 2' positions thereof, respectively.

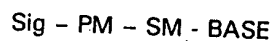
-- 566. (NEW) The oligo- or polyribonucleotide of claim 539, comprising at least one deoxyribonucleotide. --

-- 567. (NEW) The oligo- or polyribonucleotide of claim 539, having the structural formula:



wherein said Sig moiety is attached to at least one of the phosphate moieties in said structural formula. --

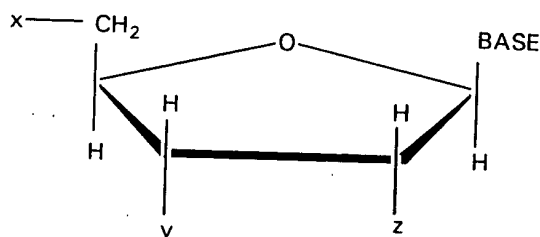
-- 568. (NEW) A composition comprising a polymeric compound having attached directly or indirectly thereto at least one deoxyribonucleotide having the formula:



wherein PM is a phosphate moiety, SM is a sugar moiety and BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, said PM being attached to SM, said BASE being attached to SM, and Sig being covalently attached to PM directly or via a chemical linkage, said Sig being a moiety capable of non-radioactive detection when attached to PM or when said deoxyribonucleotide is incorporated into said composition. --

-- 569. (NEW) The composition of claim 568, wherein said polymeric compound is selected from the group consisting of an oligo- or polynucleotide, an oligo- or polypeptide, and an oligo- or polysaccharide. --

-- 570. (NEW) A composition comprising a polymeric compound attached directly or indirectly to at least one deoxyribonucleotide having the structural formula:



wherein BASE is selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, and wherein BASE is attached to the 1' position of the pentose ring from the N1 position when BASE is a pyrimidine or from the N9 position when BASE is a purine or a deazapurine;

wherein x comprises a member selected from the group consisting of: H-, HO-, a mono-phosphate, a di-phosphate and a tri-phosphate;

wherein y comprises a member selected from the group consisting of: H-, HO-, a mono-phosphate, a di-phosphate and a tri-phosphate;

wherein z comprises H-; and

wherein Sig is covalently attached to x, y or z directly or through a chemical linkage, said Sig being a moiety capable of non-radioactive detection when so attached to x, y or z. --

-- 571. (NEW) The composition of claim 570, wherein said polymeric compound is selected from the group consisting of an oligo- or polynucleotide, an oligo- or polypeptide, and an oligo- or polysaccharide. --

-- 572. (NEW) A composition comprising a polymeric compound having attached directly or indirectly thereto at least one ribonucleotide having the formula:

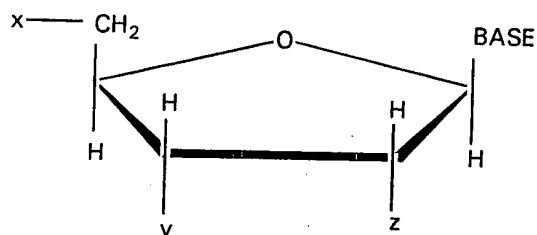
Sig - PM - SM - BASE

wherein PM is a phosphate moiety, SM is a sugar moiety and BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, said PM being attached to SM at a position of SM selected from 2', 3' and 5', or combinations thereof, said BASE being attached to SM, and Sig being covalently attached to PM directly or via a chemical linkage, said Sig being a moiety capable of non-radioactive detection when attached to PM or when said ribonucleotide is incorporated into said composition, provided that when Sig is attached through a chemical linkage to a terminal PM at the 3' position of a terminal ribonucleotide, said chemical linkage is not a cleaved 3' terminal ribonucleotide previously attached to said composition. --

-- 573. (NEW) The composition of claim 572, wherein said polymeric compound is selected from the group consisting of an oligo- or polynucleotide, an oligo- or polypeptide, and an oligo- or polysaccharide. --



-- 574. (NEW) A composition comprising a polymeric compound having attached directly or indirectly thereto at least one nucleotide having the structural formula:



wherein BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, and wherein BASE is attached to the 1' position of the pentose ring from the N1 position when BASE is a pyrimidine or from the N9 position when BASE is a purine or a deazapurine;

wherein x is selected from the group consisting of H-, HO-, a mono-phosphate, a di-phosphate and a tri-phosphate;

wherein y is selected from the group consisting of HO-, a mono-phosphate, a di-phosphate and a tri-phosphate;

wherein z is HO-; and

wherein Sig is covalently attached to x, y or z directly or through a chemical linkage, said Sig being a moiety capable of non-radioactive detection when so attached to x, y or z, provided that when Sig is attached through a chemical linkage to y of a terminal ribonucleotide, said chemical linkage is not a cleaved 3' terminal ribonucleotide previously attached to said composition. --

-- 575. (NEW) The composition of claim 572, wherein said polymeric compound is selected from the group consisting of an oligo- or polynucleotide, an oligo- or polypeptide, and an oligo- or polysaccharide. --

\* \* \* \* \*

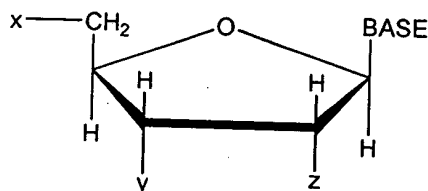
ENGELHARDT ET AL., U.S. PAT. APPL. SER. NO. 08/479,997  
AMENDMENTS TO INDEPENDENT CLAIMS 454, 482, 511 & 539  
(To Be Effected By Applicants' January 18, 2001 Amendment Under 37 C.F.R. §1.116)  
Exhibit 4 to Declaration Of Dr. Cheryl H. Agris, Attorney At Law  
(In Support Of The Written Description, Enablement & Non-Obviousness Of The  
Invention Claimed In U.S. Patent Application Serial No. 08/479,997)]

454. (Amended) An oligo- or polydeoxynucleotide which is complementary to a nucleic acid of interest or a portion thereof, said oligo- or polydeoxynucleotide comprising at least one modified nucleotide having the formula

Sig—PM—SM—BASE

wherein PM is a phosphate moiety, SM is a sugar moiety and BASE is a base moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, said PM being attached to SM, said BASE being attached to SM, and Sig being covalently attached to PM directly or through a chemical linkage, said Sig [being a moiety capable non-radioactive detection] comprising a non-radioactive label moiety which can be directly or indirectly detected when attached to PM or when said modified nucleotide is incorporated into said oligo- or polydeoxynucleotide or when said oligo- or polydeoxynucleotide is hybridized to said complementary nucleic acid of interest or a portion thereof.

482. (Amended) An oligo- or polydeoxyribonucleotide which is complementary to a nucleic acid of interest or a portion thereof, said oligo- or polydeoxyribonucleotide comprising at least one modified nucleotide having the structural formula:



wherein BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, and wherein BASE is attached to the 1' position of the pentose ring from the N1 position when BASE is a pyrimidine or from the N9 position when BASE is a purine or a deazapurine;

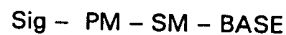
wherein x is selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate;

wherein y is selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate;

wherein z is selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate; and

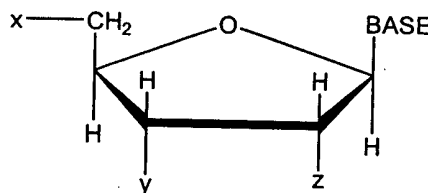
wherein Sig is covalently attached [to x, y or z] directly or through a chemical linkage to at least one phosphate selected from the group consisting of x, y, z, and a combination thereof, said Sig [being a moiety capable of non-radioactive detection] comprising a non-radioactive label moiety which can be directly or indirectly detected when so attached to [x, y or z] said phosphate or when said modified nucleotide is incorporated into said oligo- or polydeoxynucleotide or when said oligo- or polydeoxynucleotide is hybridized to said complementary nucleic acid of interest or a portion thereof.

511. (Amended) An oligo- or [polyribonucleotide] polynucleotide which is complementary to a nucleic acid of interest or a portion thereof, said oligo- or polynucleotide comprising at least one [ribonucleotide] modified nucleotide having the formula



wherein PM is a phosphate moiety, SM is a sugar moiety and BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, said PM being attached to SM [at a position of SM selected from the 2', 3' and 5' positions, or combinations thereof], said BASE being attached to SM, and Sig being covalently attached to PM directly or via a chemical linkage, said Sig [being a moiety capable of non-radioactive detection] comprising a non-radioactive label moiety which can be directly or indirectly detected when attached to PM or when said modified nucleotide is incorporated into said oligo- or [polyribonucleotide] polynucleotide, or when said oligo- or polynucleotide is hybridized to said complementary nucleic acid of interest or a portion thereof, provided that when said oligo- or polynucleotide is an oligoribonucleotide or a polyribonucleotide, and when Sig is attached through a chemical linkage to a terminal PM at the 3' position of a terminal ribonucleotide, said chemical linkage is not [a cleaved 3' terminal ribonucleotide] obtained through a 2',3' vicinal oxidation of a 3' terminal ribonucleotide previously attached to said [oligo- or polyribonucleotide] oligoribonucleotide or polyribonucleotide.

539. (Amended) An oligo- or [polyribonucleotide] polynucleotide which is complementary to a nucleic acid of interest or a portion thereof, said oligo- or polynucleotide comprising at least one modified nucleotide having the structural formula:



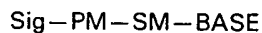
wherein BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, and wherein BASE is attached to the 1' position of the pentose ring from the N1 position when BASE is a pyrimidine or from the N9 position when BASE is a purine or a deazapurine;

wherein x is selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate;

wherein y is selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate;

wherein z is selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate; and  
 wherein Sig is covalently attached [to x, y or z] directly or through a chemical linkage to at least one phosphate selected from the group consisting of x, y and z, and a combination thereof, said Sig [being a moiety capable of non-radioactive detection] comprising a non-radioactive label moiety which can be directly or indirectly detected when so attached to [x, y or z] said phosphate or when said modified nucleotide is incorporated into said oligo- or polynucleotide, or when said oligo- or polynucleotide is hybridized to said complementary nucleic acid of interest or a portion thereof, provided that when said oligo- or polynucleotide is an oligoribonucleotide or a polyribonucleotide and when Sig is attached through a chemical linkage to [y of] a terminal PM at the 3' position of a terminal ribonucleotide, said chemical linkage is not [a cleaved 3' terminal ribonucleotide] obtained through a 2',3' vicinal oxidation of a 3' terminal ribonucleotide previously attached to said [oligo- or polyribonucleotide] oligoribonucleotide or polyribonucleotide.

454. An oligo- or polydeoxynucleotide which is complementary to a nucleic acid of interest or a portion thereof, said oligo- or polydeoxynucleotide comprising at least one modified nucleotide having the formula

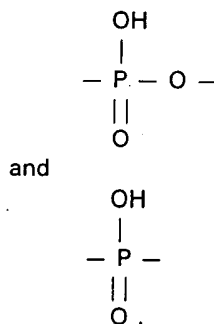


wherein PM is a phosphate moiety, SM is a sugar moiety and BASE is a base moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, said PM being attached to SM, said BASE being attached to SM, and Sig being covalently attached to PM directly or through a chemical linkage, said Sig comprising a non-radioactive label moiety which can be directly or indirectly detected when attached to PM or when said modified nucleotide is incorporated into said oligo- or polydeoxynucleotide or when said oligo- or polydeoxynucleotide is hybridized to said complementary nucleic acid of interest or a portion thereof.

455. The oligo- or polydeoxyribonucleotide of claim 454, wherein said Sig is or renders the nucleotide or the oligo- or polydeoxyribonucleotide self-signaling or self-indicating or self-detecting.

456. The oligo- or polydeoxyribonucleotide of claim 454, wherein said Sig moiety comprises at least three carbon atoms.

457. The oligo- or polydeoxyribonucleotide of claim 454, wherein said covalent attachment is selected from the group consisting of

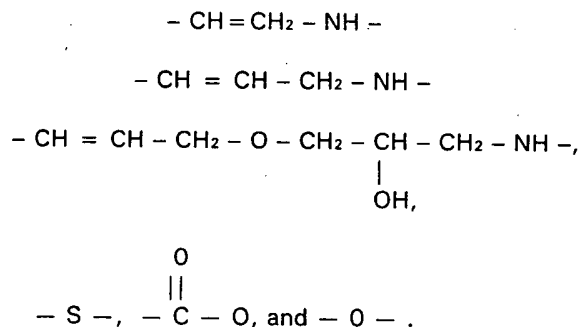


458. The oligo- or polydeoxyribonucleotide of claim 454, wherein said chemical linkage does not interfere substantially with the characteristic ability of Sig to form a detectable signal.

459. The oligo- or polydeoxyribonucleotide of claim 454, wherein said chemical linkage comprises a member selected from the group consisting of an olefinic bond at the  $\alpha$ -position relative to the point of attachment to the nucleotide, a  $-\text{CH}_2\text{NH}-$  moiety, or both.

460. The oligo- or polydeoxyribonucleotide of claim 454, wherein said chemical linkage comprises an allylamine group.

461. The oligo- or polydeoxyribonucleotide of claim 454, wherein said chemical linkage comprises or includes an olefinic bond at the  $\alpha$ -position relative to the point of attachment to the nucleotide, or any of the moieties:



462. The oligo- or polydeoxyribonucleotide of claim 454, wherein said chemical linkage of Sig includes a glycosidic linkage moiety.

463. The oligo- or polydeoxyribonucleotide of claim 454, wherein said PM is monophosphate, a diphosphate or a triphosphate and said Sig moiety is covalently attached to said PM through a phosphorus atom or phosphate oxygen.

464. The oligo- or polydeoxyribonucleotide of claim 454, wherein Sig comprises a component selected from the group consisting of biotin, iminobiotin, an electron dense component, a magnetic component, an enzyme or an enzyme component, a hormone or a hormone component, a metal-containing component, a fluorescent component, a chemiluminescent component, an antigen, a hapten and an antibody or an antibody component, or a combination of any of the foregoing.

465. The oligo- or polydeoxyribonucleotide of claim 464, wherein said electron dense component comprises ferritin.
466. The oligo- or polydeoxyribonucleotide of claim 454, wherein Sig is selected from the group consisting of a ligand and a specific ligand binding protein.
467. The oligo- or polydeoxyribonucleotide of claim 464, wherein said magnetic component comprises magnetic oxide.
468. The oligo- or polydeoxyribonucleotide of claim 467, wherein said magnetic oxide comprises ferric oxide.
469. The oligo- or polydeoxyribonucleotide of claim 464, wherein said enzyme or enzyme component is selected from the group consisting of alkaline phosphatase, acid phosphatase,  $\beta$ -galactosidase, ribonuclease, glucose oxidase and peroxidase.
470. The oligo- or polydeoxyribonucleotide of claim 464, wherein said metal-containing component is catalytic.
471. The oligo- or polydeoxyribonucleotide of claim 464, wherein said fluorescent component comprises a member selected from the group consisting of fluorescein, rhodamine and dansyl.
472. The oligo- or polydeoxyribonucleotide of claim 464, wherein Sig is selected from the group consisting of an antigen or hapten capable of complexing with an antibody or antibody component specific thereto, and an antibody or antibody component capable of complexing with an antigen or hapten.
473. The oligo- or polydeoxyribonucleotide of claim 454, wherein said oligo- or polydeoxyribonucleotide is terminally ligated or attached to a polypeptide.



474. A composition comprising the oligo- or polydeoxyribonucleotide of claim 454, a polypeptide capable of forming a complex with Sig and a moiety which can be detected when such complex is formed.

475. The composition of claim 474, wherein said polypeptide comprises polylysine.

476. The composition of claim 474, wherein said polypeptide is selected from the group consisting of avidin, streptavidin and anti-hapten immunoglobulin.

477. The composition of claim 474, wherein said Sig is a ligand and said polypeptide is an antibody thereto.

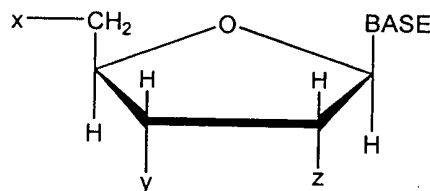
478. The oligo- or polydeoxyribonucleotide of claim 454, wherein said Sig moiety is attached to a terminal nucleotide in said oligo- or polydeoxyribonucleotide.

479. The oligo- or polydeoxyribonucleotide of claim 478, wherein the sugar moiety of said terminal nucleotide has a hydrogen atom at the 2' position thereof.

480. The oligo- or polydeoxyribonucleotide of claim 478, wherein the sugar moiety of said terminal nucleotide has oxygen atoms at each of the 2' and 3' positions thereof.

481. The oligo- or polydeoxyribonucleotide of claim 454, comprising at least one ribonucleotide.

482. An oligo- or polydeoxyribonucleotide which is complementary to a nucleic acid of interest or a portion thereof, said oligo- or polydeoxyribonucleotide comprising at least one modified nucleotide having the structural formula:



wherein BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, and wherein BASE is attached to the 1' position of the pentose ring from the N1 position when BASE is a pyrimidine or from the N9 position when BASE is a purine or a deazapurine;

wherein x is selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate;

wherein y is selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate;

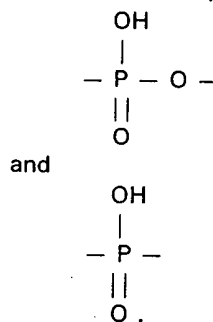
wherein z is selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate; and

wherein Sig is covalently attached directly or through a chemical linkage to at least one phosphate selected from the group consisting of x, y, z, and a combination thereof, said Sig comprising a non-radioactive label moiety which can be directly or indirectly detected when so attached to said phosphate or when said modified nucleotide is incorporated into said oligo- or polydeoxynucleotide or when said oligo- or polydeoxynucleotide is hybridized to said complementary nucleic acid of interest or a portion thereof.

483. The oligo- or polydeoxyribonucleotide of claim 482, wherein said Sig is or renders the nucleotide or the oligo- or polydeoxyribonucleotide self-signaling or self-indicating or self-detecting.

484. The oligo- or polydeoxyribonucleotide of claim 482, wherein said Sig moiety comprises at least three carbon atoms.

485. The oligo- or polydeoxyribonucleotide of claim 482, wherein said covalent attachment is selected from the group consisting of

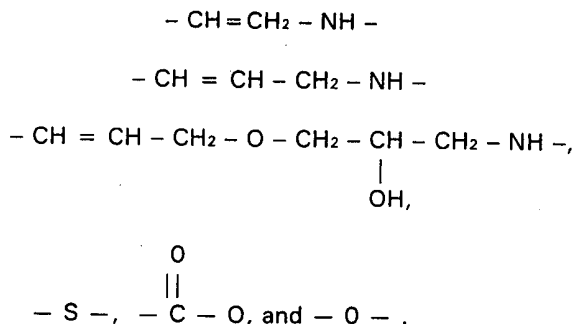


486. The oligo- or polydeoxyribonucleotide of claim 482, wherein said chemical linkage does not interfere substantially with the characteristic ability of Sig to form a detectable signal.

487. The oligo- or polydeoxyribonucleotide of claim 482, wherein said chemical linkage comprises a member selected from the group consisting of an olefinic bond at the  $\alpha$ -position relative to the point of attachment to the nucleotide, a  $-\text{CH}_2\text{NH}-$  moiety, or both.

488. The oligo- or polydeoxyribonucleotide of claim 482, wherein said chemical linkage comprises an allylamine group.

489. The oligo- or polydeoxyribonucleotide of claim 482, wherein said chemical linkage comprises or includes an olefinic bond at the  $\alpha$ -position relative to the point of attachment to x, y or z, or any of the moieties:



490. The oligo- or polydeoxyribonucleotide of claim 482, wherein said chemical linkage of Sig includes a glycosidic linkage moiety.
491. The oligo- or polydeoxyribonucleotide of claim 482, wherein said x and y each comprise a member selected from the group consisting of a monophosphate, a diphosphate and a triphosphate and said Sig moiety is covalently attached to either or both of said x and y a phosphorus atom or phosphate oxygen.
492. The oligo- or polydeoxyribonucleotide of claim 482, wherein Sig comprises a component selected from the group consisting of biotin, iminobiotin, an electron dense component, a magnetic component, an enzyme or an enzyme component, a hormone or a hormone component, a metal-containing component, a fluorescent component, a chemiluminescent component, an antigen, a hapten and an antibody or an antibody component, or a combination of any of the foregoing.
493. The oligo- or polydeoxyribonucleotide of claim 492, wherein said electron dense component comprises ferritin.
494. The oligo- or polydeoxyribonucleotide of claim 482, wherein Sig is selected from the group consisting of a ligand and a specific ligand binding protein.
495. The oligo- or polydeoxyribonucleotide of claim 492, wherein said magnetic component comprises magnetic oxide.
496. The oligo- or polydeoxyribonucleotide of claim 495, wherein said magnetic oxide comprises ferric oxide.
497. The oligo- or polydeoxyribonucleotide of claim 492, wherein said enzyme or enzyme component is selected from the group consisting of alkaline phosphatase, acid phosphatase,  $\beta$ -galactosidase, ribonuclease, glucose oxidase and peroxidase.
498. The oligo- or polydeoxyribonucleotide of claim 492, wherein said metal-containing component is catalytic.

499. The oligo- or polydeoxyribonucleotide of claim 492, wherein said fluorescent component comprises a member selected from the group consisting of fluorescein, rhodamine and dansyl.

500. The oligo- or polydeoxyribonucleotide of claim 492, wherein Sig is selected from the group consisting of an antigen or hapten capable of complexing with an antibody or antibody component specific thereto, and an antibody or antibody component capable of complexing with an antigen or hapten.

501. The oligo- or polydeoxyribonucleotide of claim 482, wherein said oligo- or polydeoxyribonucleotide is terminally ligated or attached to a polypeptide.

502. A composition comprising the oligo- or polydeoxyribonucleotide of claim 482, a polypeptide capable of forming a complex with Sig and a moiety which can be detected when such complex is formed.

503. The composition of claim 500, wherein said polypeptide comprises polylysine.

504. The composition of claim 502, wherein said polypeptide is selected from the group consisting of avidin, streptavidin and anti-hapten immunoglobulin.

505. The composition of claim 502, wherein said Sig is a ligand and said polypeptide is an antibody thereto.

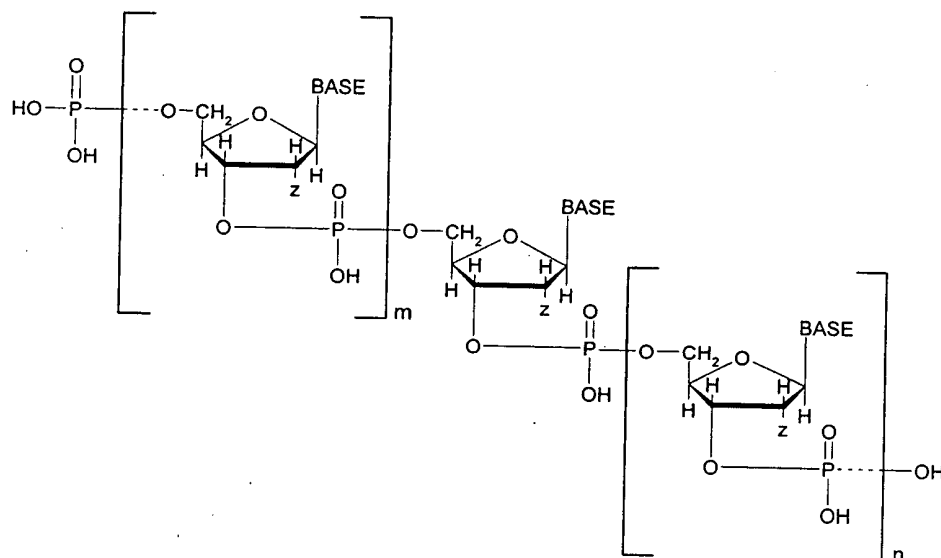
506. The oligo- or polydeoxyribonucleotide of claim 482, wherein said Sig moiety is attached to a terminal nucleotide in said oligo- or polydeoxyribonucleotide.

507. The oligo- or polydeoxyribonucleotide of claim 506, wherein z of said terminal nucleotide comprises a hydrogen atom at the 2' position thereof.

508. The oligo- or polydeoxyribonucleotide of claim 506, wherein both y and z of said terminal nucleotide comprise an oxygen atom at each of the 3' and 2' positions thereof, respectively.

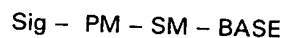
509. The oligo- or polydeoxyribonucleotide of claim 482, comprising at least one ribonucleotide.

510. The oligo- or polydexoxyribonucleotide of claim 482, having the structural formula:



, wherein m and n represent integers from 0 up to about 100,000, and wherein said Sig moiety is attached to at least one of the phosphate moieties in said structural formula.

511. An oligo- or polynucleotide which is complementary to a nucleic acid of interest or a portion thereof, said oligo- or polynucleotide comprising at least one modified nucleotide having the formula

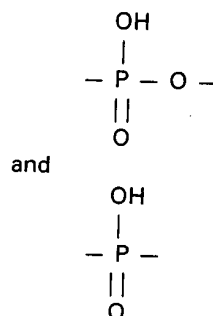


wherein PM is a phosphate moiety, SM is a sugar moiety and BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, said PM being attached to SM, said BASE being attached to SM, and Sig being covalently attached to PM directly or via a chemical linkage, said Sig comprising a non-radioactive label moiety which can be directly or indirectly detected when attached to PM or when said modified nucleotide is incorporated into said oligo- or polynucleotide, or when said oligo- or polynucleotide is hybridized to said complementary nucleic acid of interest or a portion thereof, provided that when said oligo- or polynucleotide is an oligoribonucleotide or a polyribonucleotide, and when Sig is attached through a chemical linkage to a terminal PM at the 3' position of a terminal ribonucleotide, said chemical linkage is not obtained through a 2',3' vicinal oxidation of a 3' terminal ribonucleotide previously attached to said oligoribonucleotide or polyribonucleotide.

512. The oligo- or polynucleotide of claim 511, wherein said Sig is or renders the nucleotide or the oligo- or polynucleotide self-signaling or self-indicating or self-detecting.

513. The oligo- or polynucleotide of claim 511, wherein said Sig moiety comprises at least three carbon atoms.

514. The oligo- or polynucleotide of claim 511, wherein said covalent attachment is selected from the group consisting of

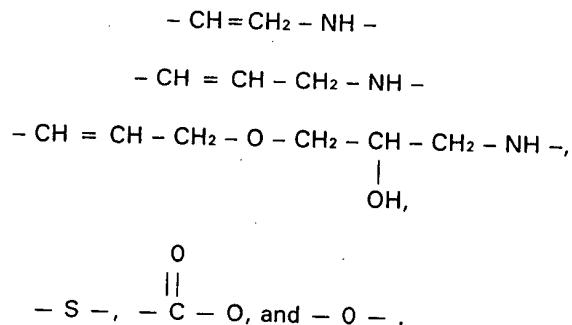


515. The oligo- or polynucleotide of claim 511, wherein said chemical linkage does not interfere substantially with the characteristic ability of Sig to form a detectable signal.

516. The oligo- or polynucleotide of claim 511, wherein said chemical linkage comprises a member selected from the group consisting of an olefinic bond at the  $\alpha$ -position relative to the point of attachment to the nucleotide, a  $-\text{CH}_2\text{NH}-$  moiety, or both.

517. The oligo- or polynucleotide of claim 511, wherein said chemical linkage comprises an allylamine group.

518. The oligo- or polynucleotide of claim 511, wherein said chemical linkage comprises or includes an olefinic bond at the  $\alpha$ -position relative to the point of attachment to the nucleotide, or any of the moieties:



519. The oligo- or polynucleotide of claim 511, wherein said chemical linkage of Sig includes a glycosidic linkage moiety.

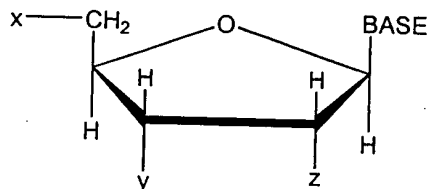
520. The oligo- or polynucleotide of claim 511, wherein said PM is a monophosphate, a diphosphate or a triphosphate and said Sig moiety is covalently attached to said PM through a phosphorus atom or a phosphate oxygen.



521. The oligo- or polynucleotide of claim 511, wherein Sig comprises a component selected from the group consisting of biotin, iminobiotin, an electron dense component, a magnetic component, an enzyme or an enzyme component, a hormone or a hormone component, a metal-containing component, a fluorescent component, a chemiluminescent component, an antigen, a hapten and an antibody or an antibody component, or a combination of any of the foregoing.
522. The oligo- or polynucleotide of claim 521, wherein said electron dense component comprises ferritin.
523. The oligo- or polynucleotide of claim 511, wherein Sig is selected from the group consisting of a ligand and a specific ligand binding protein.
524. The oligo- or polynucleotide of claim 521, wherein said magnetic component comprises magnetic oxide.
525. The oligo- or polynucleotide of claim 524, wherein said magnetic oxide comprises ferric oxide.
526. The oligo- or polynucleotide of claim 521, wherein said enzyme or enzyme component is selected from the group consisting of alkaline phosphatase, acid phosphatase,  $\beta$ -galactosidase, ribonuclease, glucose oxidase and peroxidase.
527. The oligo- or polynucleotide of claim 521, wherein said metal-containing component is catalytic.
528. The oligo- or polynucleotide of claim 521, wherein said fluorescent component comprises a member selected from the group consisting of fluorescein, rhodamine and dansyl.
529. The oligo- or polynucleotide of claim 521, wherein Sig is selected from the group consisting of an antigen or hapten capable of complexing with an antibody or antibody component specific thereto, and an antibody or antibody component capable of complexing with an antigen or hapten.

530. The oligo- or polynucleotide of claim 511, wherein said oligo- or polynucleotide is terminally ligated or attached to a polypeptide.
531. A composition comprising the oligo- or polynucleotide of claim 511, a polypeptide capable of forming a complex with Sig and a moiety which can be detected when such complex is formed.
532. The composition of claim 531, wherein said polypeptide comprises polylysine.
533. The composition of claim 531, wherein said polypeptide is selected from the group consisting of avidin, streptavidin and anti-hapten immunoglobulin.
534. The composition of claim 531, wherein said Sig is a ligand and said polypeptide is an antibody thereto.
535. The oligo- or polynucleotide of claim 511, wherein said Sig moiety is attached to a terminal nucleotide in said oligo- or polynucleotide.
536. The oligo- or polynucleotide of claim 535, wherein the sugar moiety of said terminal nucleotide has a hydrogen atom at the 2' position thereof.
537. The oligo- or polynucleotide of claim 535, wherein the sugar moiety of said terminal nucleotide has an oxygen atom at each of the 2' and 3' positions thereof.
538. The oligo- or polynucleotide of claim 511, comprising at least one deoxyribonucleotide.

539. An oligo- or polynucleotide which is complementary to a nucleic acid of interest or a portion thereof, said oligo- or polynucleotide comprising at least one modified nucleotide having the structural formula:



wherein BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, and wherein BASE is attached to the 1' position of the pentose ring from the N1 position when BASE is a pyrimidine or from the N9 position when BASE is a purine or a deazapurine;

wherein x is selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate;

wherein y is selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate;

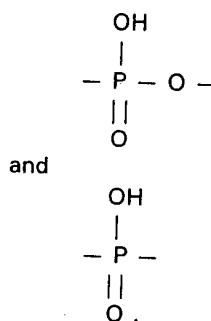
wherein z is selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate; and

wherein Sig is covalently attached directly or through a chemical linkage to at least one phosphate selected from the group consisting of x, y and z, and a combination thereof, said Sig comprising a non-radioactive label moiety which can be directly or indirectly detected when so attached to said phosphate or when said modified nucleotide is incorporated into said oligo- or polynucleotide, or when said oligo- or polynucleotide is hybridized to said complementary nucleic acid of interest or a portion thereof, provided that when said oligo- or polynucleotide is an oligoribonucleotide or a polyribonucleotide and when Sig is attached through a chemical linkage to a terminal PM at the 3' position of a terminal ribonucleotide, said chemical linkage is not obtained through a 2',3' vicinal oxidation of a 3' terminal ribonucleotide previously attached to said oligoribonucleotide or polyribonucleotide.

540. The oligo- or polynucleotide of claim 539, wherein said Sig is or renders the nucleotide or the oligo- or polynucleotide self-signaling or self-indicating or self-detecting.

541. The oligo- or polynucleotide of claim 539, wherein said Sig moiety comprises at least three carbon atoms.

542. The oligo- or polynucleotide of claim 539, wherein said covalent attachment is selected from the group consisting of

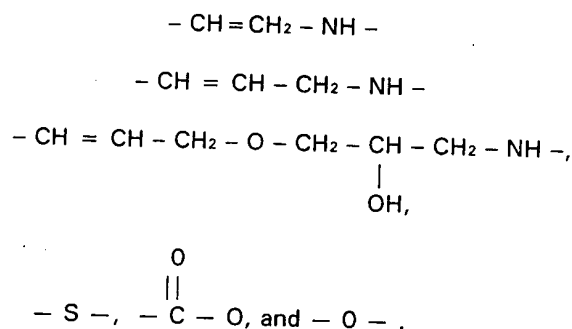


543. The oligo- or polynucleotide of claim 539, wherein said chemical linkage does not interfere substantially with the characteristic ability of Sig to form a detectable signal.

544. The oligo- or polynucleotide of claim 539, wherein said chemical linkage comprises a member selected from the group consisting of an olefinic bond at the  $\alpha$ -position relative to the point of attachment to the nucleotide, a  $-\text{CH}_2\text{NH}-$  moiety, or both.

545. The oligo- or polynucleotide of claim 539, wherein said chemical linkage comprises an allylamine group.

546. The oligo- or polynucleotide of claim 539, wherein said chemical linkage comprises or includes an olefinic bond at the  $\alpha$ -position relative to x, y or z, or any of the moieties:



547. The oligo- or polynucleotide of claim 539, wherein said chemical linkage of Sig includes a glycosidic linkage moiety.

548. The oligo- or polynucleotide of claim 539, wherein said x and y each comprise a member selected from the group consisting of a monophosphate, a diphosphate and a triphosphate and Sig moiety is covalently attached to either or both of said x and y a phosphorus atom or a phosphate oxygen.

549. The oligo- or polynucleotide of claim 539, wherein Sig comprises a component selected from the group consisting of biotin, iminobiotin, an electron dense component, a magnetic component, an enzyme or an enzyme component, a hormone or a hormone component, a metal-containing component, a fluorescent component, a chemiluminescent component, an antigen, a hapten and an antibody or an antibody component, or a combination of any of the foregoing.

550. The oligo- or polynucleotide of claim 549, wherein said electron dense component comprises ferritin.

551. The oligo- or polynucleotide of claim 539, wherein Sig is selected from the group consisting of a ligand and a specific ligand binding protein.

552. The oligo- or polynucleotide of claim 549, wherein said magnetic component comprises magnetic oxide.

553. The oligo- or polynucleotide of claim 552, wherein said magnetic oxide comprises ferric oxide.
554. The oligo- or polynucleotide of claim 549, wherein said enzyme or enzyme component is selected from the group consisting of alkaline phosphatase, acid phosphatase,  $\beta$ -galactosidase, ribonuclease, glucose oxidase and peroxidase.
555. The oligo- or polynucleotide of claim 549, wherein said metal-containing component is catalytic.
556. The oligo- or polynucleotide of claim 549, wherein said fluorescent component comprises a member selected from the group consisting of fluorescein, rhodamine and dansyl.
557. The oligo- or polynucleotide of claim 549, wherein Sig is selected from the group consisting of an antigen or hapten capable of complexing with an antibody or antibody component specific thereto, and an antibody or antibody component capable of complexing with an antigen or hapten.
558. The oligo- or polynucleotide of claim 539, wherein said oligo- or polynucleotide is terminally ligated or attached to a polypeptide.
559. A composition comprising the oligo- or polynucleotide of claim 539, a polypeptide capable of forming a complex with Sig and a moiety which can be detected when such complex is formed.
560. The composition of claim 559, wherein said polypeptide comprises polylysine.
561. The composition of claim 559, wherein said polypeptide is selected from the group consisting of avidin, streptavidin and anti-hapten immunoglobulin.
562. The composition of claim 559, wherein said Sig is a ligand and said polypeptide is an antibody thereto.

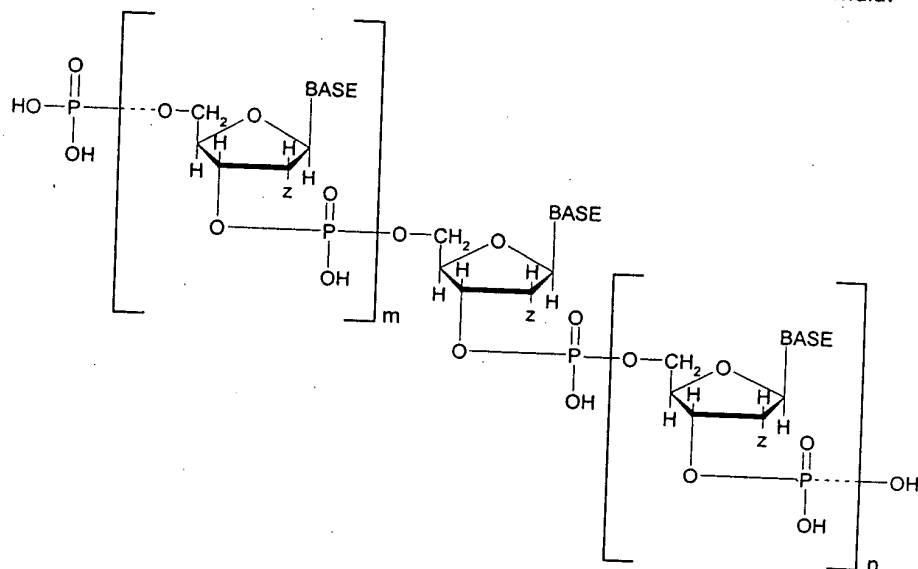
563. The oligo- or polynucleotide of claim 539, wherein said Sig moiety is attached to a terminal nucleotide in said oligo- or polynucleotide.

564. The oligo- or polynucleotide of claim 563, wherein z of said terminal nucleotide comprises a hydrogen atom at the 2' position thereof.

565. The oligo- or polynucleotide of claim 563, wherein both y and z of said terminal nucleotide comprise an oxygen atom at each of the 3' and 2' positions thereof, respectively.

566. The oligo- or polynucleotide of claim 539, comprising at least one deoxyribonucleotide.

567. The oligo- or polynucleotide of claim 539, having the structural formula:



, wherein m and n represent integers from 0 up to about 100,000, and wherein said Sig moiety is attached to at least one of the phosphate moieties in said structural formula.

\* \* \* \* \*

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicants:	Dean L. Engelhardt et al.)	
Serial No.	08/479,997	Group Art Unit: 1809
Filed:	June 7, 1995	Examiner: Scott Houtteman
Title:	A PHOSPHATE MOIETY LABELED NUCLEOTIDE, AND AN OLIGO- OR POLYNU- CLEOTIDE, AND OTHER COMPOSITIONS COMPRISING SUCH PHOSPHATE MOIETY LABELED NUCLEOTIDES	

527 Madison Avenue, 9th Floor  
New York, New York 10022

Honorable Commissioner of Patents and Trademarks  
The United States Patent and Trademark Office  
Washington, D.C. 20231

**DECLARATION OF DR. DEAN L. ENGELHARDT  
IN SUPPORT OF ADEQUATE DESCRIPTION AND ENABLEMENT**

I, Dean L. Engelhardt, hereby declare as follows:

1. I am the Dean L. Engelhardt who is named as an applicant on the above-identified application. I am a co-inventor of the subject matter claimed in this application. Furthermore, I am familiar with the contents of this application.
2. I am currently employed by Enzo Biochem, Inc., 527 Madison Avenue, New York, New York 10022 as Senior Vice President, having held that position since 1988. Prior to my employment at Enzo Biochem, Inc., I was Associate Professor of Microbiology at Columbia University College of Physicians and Surgeons, New York City, having earlier obtained my doctorate from Rockefeller University in New York City.

Enz-5(D6)(C2)



3. In addition to my position as Senior Vice President of Enzo Biochem, Inc., I have also served as Director of Research in which capacity I have overseen scientific research activities for the company and its subsidiaries. I also continue to oversee various research projects. Among my responsibilities at Enzo Biochem, Inc. have been the development of new nucleic acid technology and hybridization formats, including new diagnostic and therapeutic approaches and agents based upon nucleic acid technology.

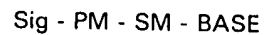
4. I understand that the presently pending claims in this application are directed to a nucleotide in which a moiety Sig is covalently attached to the phosphate moiety (a di-phosphate or tri-phosphate) directly or via a chemical linkage. Sig is capable of non-radioactive detection when attached to the phosphate or when the nucleotide is incorporated into an oligo- or polynucleotide or other composition. I further understand that other presently pending claims are directed to an oligo- or polynucleotide and to other compositions including those comprising a polymeric compound - all of which comprise at least one nucleotide in which a moiety Sig capable of non-radioactive detection is attached to the phosphate moiety thereof directly or via a chemical linkage.

5. In further detail, I understand that the presently pending claims are directed to the just-described nucleotide (278-301, 308-309, 373-394 and 401-404), the oligo- or polynucleotide (310-337 and 405-432), and other compositions (303-307, 338-372 and 433-453) comprising phosphate-modified nucleotides in which a moiety Sig capable of non-radioactive detection is attached thereto directly or via a chemical linkage.

A. Claim 278 is independent and defines the nucleotide as having the formula  
Sig - PM - SM - BASE  
wherein PM is selected from a di-phosphate or a tri-phosphate moiety, SM is a sugar moiety and BASE is a moiety selected from a pyrimidine, a purine and a deazapurine, or analog thereof. PM is attached to SM and BASE is attached to SM. Sig is covalently attached to PM directly or via a chemical linkage and it represents a moiety capable of non-radioactive detection when attached to PM. Furthermore, the claimed nucleotide is defined as being capable of incorporation into an oligo- or polynucleotide. Other embodiments of the aforementioned nucleotide include those defining the self-signaling or self-indicating or self-detecting nature of Sig (claim 279); the Sig moiety comprising at least three carbon atoms (claim 280); the

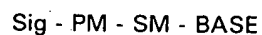
covalent attachment or chemical linkage of Sig to PM (claims 281-287); specific members of Sig (claim 288-301); and the nucleotide comprising a deoxyribonucleotide (claim 308) and a ribonucleotide (claim 309).

B. I also understand that the presently pending claims define an oligo- or polynucleotide comprising at least one such phosphate-modified nucleotide, the oligo- or polynucleotide being terminally ligated or attached to a polypeptide (claim 302). The claims also include other compositions comprising an oligo- or polynucleotide including at least one such phosphate-modified nucleotide and a polypeptide capable of forming a complex with Sig and a moiety which can be detected when the complex is formed (claims 303-307). My understanding of the present claims extend to the oligo- or polynucleotide of which claim 310 is independent and arguably the broadest. Claim 310 defines the oligo- or polynucleotide as comprising at least one nucleotide having the formula



wherein PM is a phosphate moiety, SM is a sugar moiety and BASE is a moiety selected from a pyrimidine, a purine and a deazapurine, or analog thereof. PM is attached to SM and BASE is attached to SM. Sig is covalently attached to PM directly or via a chemical linkage and represents a moiety capable of non-radioactive detection when attached to PM or when the nucleotide is incorporated into the oligo- or polynucleotide. Other dependent claims directed to this oligo- or polynucleotide include embodiments of the self-signaling or self-indicating or self-detecting nature of Sig (claim 311); Sig as comprising at least three carbon atoms (claim 312); the covalent attachment or chemical linkage of Sig to PM (claim 313-320); specific members of Sig (claims 321-334); the attachment of Sig to a terminal nucleotide (claims 335-337); and the nucleotide comprising a deoxyribonucleotide (claim 427) and a ribonucleotide (claim 428).

C. My understanding of the presently pending claims also extend to the composition comprising a polymeric compound having attached directly or indirectly thereto at least one nucleotide having the formula:



wherein PM is a phosphate moiety, SM is a sugar moiety and BASE is a moiety selected from a pyrimidine, purine and a deazapurine, or analog thereof. PM is attached to SM, BASE is attached to SM, and Sig is covalently attached to PM directly or via a chemical linkage and it represents a moiety capable of non-

radioactive detection when attached to PM and when the nucleotide is incorporated into such composition. Claim 338 is independent and is arguably the broadest such composition. Other dependent claims are directed to embodiments describing the self-signaling or self-indicating or self-detecting nature of Sig (claim 339); Sig as comprising at least three carbon atoms (claim 340); the covalent attachment or chemical linkage of Sig to PM (claims 341-348); specific members of Sig (claims 349-361); the composition and a complex-forming polypeptide (claims 363-368); and specific members of the polymeric component (claims 369-372).

D. I also understand that other claims are pending in which the aforementioned moieties (BASE, PM and Sig) are attached to a pentose ring sugar moiety (SM) given by a structural formula set forth in these other claims. The claims reciting such a structural formula include those that are directed to a phosphate-modified (a di-phosphate or tri-phosphate) nucleotide (claims 373-394 and 401-404); an oligo- or polynucleotide or other composition comprising at least one such nucleotide (claims 395-400); an oligo- or polynucleotide comprising at least one phosphate-modified nucleotide (claims 405-432); and a composition comprising a polymeric compound attached directly or indirectly to at least one such phosphate-modified nucleotide (claims 433-453).

6. I have read the two Office Actions dated June 20, 1996 and June 25, 1997 that were issued in connection with this application. I understand that in both Office Actions the specification of this application was objected to and the claims were rejected for lack of adequate description and enablement.

A. The Examiner's position on the description issue taken from the June 20, 1996 Office Action is as follows:

Claims 207-224 and 227-262 are drawn to nucleotides having the "Sig" moiety attached to the phosphate moiety wherein the Sig moiety is limited to one of several molecular classes such as "at least three carbon atoms, a glycosidic linkage moiety, biotin, iminobiotin, ferritin, an antigen, a hapten, an antibody, etc.

Support for these claims was pointed out in original claims 125, 41, 84, 126, 129, 127 and 128. However, these claims are drawn to nucleotides in which the "Sig" moiety is attached to the base.

The only support that was found in the original disclosure was in a passage on pages 96-97 which begins "By way of summary." This passage defines "Sig" as binding to either base, sugar or phosphate and then defines "Sig" to include the particular products in

the newly presented claims. However, there is no explicit description of the various claimed products bound to the phosphate anywhere in the specification. In contrast, the base-linked "Sig" moieties have numerous complex chemical reactions which are necessary to synthesis the various products. These reactions include various solvents, reactants and protecting groups which are necessary so that only the base was modified and not the reactive groups on the sugar or phosphates. Thus, an explicit description of the "phosphate-Sig" reactions would have been expected in order for a skilled artisan to have reasonably concluded that the original disclosure evidenced "possession" of the currently claimed invention.

Thus, in view of the phrase "by way of summary" and the absence of any "phosphate-Sig" reactions to summarize; and in view of the complex nature of these reactions, the skilled artisan would not have reasonably expected this specification to put the artisan in possession of the invention as now claimed.

Since support for these claims was not found where pointed out nor elsewhere in the specification, these claims are considered "new matter."

B. The Examiner's position on enablement as set forth in the June 20, 1996 Office Action is as follows:

Claims 204-224 and 227-262 are broadly drawn to nucleotides having various "Sig moieties" attached to the phosphate moiety.

The specification contains a sufficiently detailed disclosure, such as in Examples I-VII, to enable the construction of "sig-base" nucleotides, that is nucleotides in which the "Sig" moiety is linked to the base. It is noted that these reactions contain many specific solvents, reactants and protecting groups. This detailed disclosure enables one to obtain a reasonable product yield, a product of suitable stability for its intended use in nucleic acid detection assays and a product reasonably free of unwanted side products in which the Sig moiety is attached at the wrong places on the nucleotide.

However, there is no analogous disclosure for the attachment of the "Sig-phosphate" nucleotides. The broadly claimed "Sig moieties" include a very diverse population of molecules, from simple inorganic compounds like radioactive cobalt to the complex organic molecules like enzymes. Accordingly, there are a vast number of possible chemical reaction schemes one could attempt. Without specific guidance or examples, the skilled artisan would expect that the vast majority of these reaction schemes would fail. Either the product yields would be low, the products would be too unstable or the products would be too hard to purify away from extraneous side products.

It is difficult to predict the behavior of a complex organic molecule with numerous functional groups: primary amines, carboxyl groups and alcohol groups. There is no way to establish, before the fact, which reaction conditions will result in high yields and stable products that can be purified from extraneous byproducts.

The level of skill is high in this field. Nevertheless, in view of the large scope of these claims, the lack of any guidance or specific

examples, the high degree of unpredictability, the complex nature of the invention which requires both inorganic and organic chemical syntheses; it would have required undue experimentation to enable a reasonable number of embodiments within the scope of these claims.

C. I also understand that the enablement issue was maintained by the Examiner in the most recent June 25, 1997 Office Action, the Examiner stating there:

Applicant argues that Example V, citing Halloran et al., describes labeling of the phosphate moiety with "Sig." This argument is not persuasive for two reasons.

First, essential subject matter cannot be incorporated by reference to a research article. Second, the claims are not limited to a carbodiimide mediated linking of proteins to nucleotides but read generally on any "Sig" linked to the phosphate moiety by any method. Thus, the scope of the described subject matter is very different from the scope of the claimed subject matter. This difference in scope is reflected in the response filed 12/20/96, page 6, first paragraph: "[I]t is evident that at least one means of coupling nucleotide and oligonucleotides to labels through the phosphate moiety was available . . ." Since support for the subject matter of the same scope was not found, nor was it pointed out, the rejection under 35 U.S.C. § 112, first paragraph, description requirement is MAINTAINED.

Applicant argues that the claims of US Pat. 5,260,433 is evidence of descriptive support and enablement for the present claims. This argument is not persuasive. Each case is argued on its own merits. Any arguments made in other cases must be made of record in this case in order to be considered.

7. It is my opinion that the originally filed specification does indeed support the subject matter of the pending claims which are adequately described to the point that a skilled artisan would have reasonably concluded that the original disclosure evidenced possession of the invention currently being claimed. It is also my opinion that the specification provides a disclosure sufficiently enabling so that the skilled artisan, armed with the disclosure and knowledge in the art at the time the application was originally filed in 1982, would have been able to practice the claimed invention without excessive experimentation, or to put it in other words, to practice the invention with a minimum of experimentation. I am making this Declaration to substantiate both the support and adequate description in the specification for the claims and the enabling nature of the specification.

8. A. With respect to the support and description in the specification for the presently claimed invention, I offer the following remarks. In order to understand the basis of this invention, it would be helpful to describe briefly the state of

technology with respect to nucleic acid labeling and detection in the early 1980s. In 1981, Dr. David C. Ward and his group at Yale became textbook celebs for their discovery that nucleotides could be non-radioactively labeled in the so-called non-disruptive positions of the base without substantially interfering with the capability of the labeled nucleotide to be incorporated into an oligo- or polynucleotide, and without substantially interfering with the capability of the oligo- or polynucleotide to be detected by means of the labeled nucleotide that was incorporated. Prior to 1981, nucleic acids were conventionally labeled with radioactive isotopes, most notably  $^{32}\text{P}$ . With Dr. Ward's discovery, the world turned en masse to non-radioactive labeling of nucleic acids, that discovery culminating in the issuance of several United States and foreign patents including the following: U.S. Patent Nos. 4,711,955; 5,328,824; 5,449,767; 5,476,928; and European Patent Nos. 0 063 879 B1 and 0 329 198 A2. The latter is an allowed application that has not yet been formally granted.

B. The principles or criteria behind the Ward discovery are exquisitely set forth in their patent specifications. In U.S. Patent No. 5,328,824, for example, the Ward "criteria" for base labeling nucleotides are described in columns 6 and 7 under the section titled "DETAILED DESCRIPTION OF THE INVENTION:"

Several essential criteria must be satisfied in order for a modified nucleotide to be generally suitable as a substitute for a radioactively-labeled form of a naturally occurring nucleotide. First, the modified compound must contain a substituent or probe that is unique, i.e., not normally found associated with nucleotides or polynucleotides. Second, the probe must react specifically with chemical or biological reagents to provide a sensitive detection system. Third, the analogs must be relatively efficient substrates for commonly studied nucleic acid enzymes, since numerous practical applications require that the analog be enzymatically metabolized, e.g., the analogs must function as substrates for nucleic acid polymerases. For this purpose, probe moieties should not be placed on ring positions that sterically, or otherwise, interfere with the normal Watson-Crick hydrogen bonding potential of the bases. Otherwise, the substituents will yield compounds that are inactive as polymerase substrates. Substitution at ring positions that alter the normal "anti" nucleoside conformation also must be avoided since such conformational changes usually render nucleotide derivatives unacceptable as polymerase substrates. Normally, such considerations limit substitution positions to the 5-position of a pyrimidine and the 7-position of a purine or a 7-deazapurine.

Fourth, the detection system should be capable of interacting with probe substituents incorporated into both single-stranded and double-stranded polynucleotides in order to be compatible with nucleic

acid hybridization methodologies. To satisfy this criterion, it is preferable that the probe moiety be attached to the purine or pyrimidine through a chemical linkage or "linker arm" so that it can readily interact with antibodies, other detector proteins, or chemical reagents.

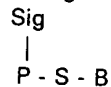
Fifth, the physical and biochemical properties of polynucleotides containing small numbers of probe substituents should not be significantly altered so that current procedures using radioactive hybridization probes need not be extensively modified. This criterion must be satisfied whether the probe is introduced by enzymatic or direct chemical means.

Finally, the linkage that attaches the probe moiety should withstand all experimental conditions to which normal nucleotides and polynucleotides are routinely subjected, e.g., extended hybridization times at elevated temperatures, phenol and organic solvent extraction, electrophoresis, etc.

All of these criteria are satisfied by the modified nucleotides described herein.

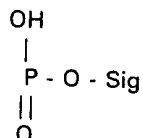
9. A. A short time after Ward's discovery, it was unexpectedly discovered by the instant inventors - all of whom were scientists at Enzo - that nucleic acid labeling and detection could be extended far beyond, but moreover, in total contradiction to Ward's discovery and criteria. Our subsequent and unexpected discovery that culminated in the filing of the first application in the family in 1982 flew headlong against Ward because the positions for labeling the nucleic acid now involved the so-called "disruptive" and "semi-disruptive" positions in the base. Moreover, the novel labeling and labeled compositions involved not only such positions in the base moiety, but the sugar and phosphate moieties as well. This discovery with respect to the phosphate moiety is set forth in several portions in the instant specification. In the specification, page 94, last paragraph, and continuing through page 95, first paragraph, the phosphate-modified nucleotides and compositions of the present invention are specifically disclosed but not for the first time:

Still further, nucleotides in accordance with the practices of this invention include the nucleotides having the formula,



wherein P is the phosphoric acid moiety, S the sugar moiety and B the base moiety. In these special nucleotides the P moiety is attached to the 3' and/or the 5' position of the S moiety when the nucleotide is deoxyribonucleotide and at the 2', 3' and/or 5' position when the nucleotide is a ribonucleotide. The base B is either a purine or a pyrimidine and the B moiety is attached from the N1 or the N9 position to the 1' position of the sugar moiety when said B moiety is a

pyrimidine or a purine, respectively. The Sig chemical moiety is covalently attached to the phosphoric acid P moiety via the chemical linkage



said Sig, when attached to said P moiety being capable of signalling itself or making itself self-detecting or its presence known and desirably the nucleotide is capable of being incorporated into a double-stranded or DNA, RNA or DNA-RNA hybrid.

Later on page 96, and continuing through the first paragraph on page 98, further description of the present invention is amply provided:

By way of summary, as indicated hereinabove with respect to the make-up of the various special nucleotides in accordance with this invention, the special nucleotides can be described as comprising a phosphoric acid moiety P, a sugar moiety S and a base moiety B, a purine or pyrimidine, which combination of P-S-B is well known with respect to and defines nucleotides, both deoxyribinucleotides and ribonucleotides. The nucleotides are then modified in accordance with the practices of this invention by having covalently attached thereto, to the P moiety and/or the S moiety and/or the B moiety, a chemical moiety Sig. The chemical moiety Sig so attached to the nucleotide P-S-B is capable of rendering or making the resulting nucleotide, now comprising P-S-B with the Sig moiety being attached to one or more of the other moieties, self-detecting or signalling itself or capable of making its presence known per se, when incorporated into a polynucleotide, especially a double-stranded polynucleotide, such as double-stranded DNA, a double-stranded RNA or a double-stranded DNA-RNA hybrid. The Sig moiety desirably should not interfere with the capability of the nucleotide to form a double-stranded polynucleotide containing the special Sig-containing nucleotide in accordance with this invention and, when so incorporated therein, the Sig-containing nucleotide is capable of detection, localization or observation.

The Sig moiety employed in the make-up of the special nucleotides of this invention could comprise an enzyme or enzymic material, such as alkaline phosphatase, glucose oxidase, horseradish peroxidase or ribonuclease. The Sig moiety could also contain a fluorescing component, such as fluorescein or rhodamine or dansyl. If desired, the Sig moiety could include a magnetic component associated or attached thereto, such as a magnetic oxide or magnetic iron oxide, which would make the nucleotide or polynucleotide containing such a magnetic-containing Sig moiety detectable by magnetic means. The Sig moiety might also include an electron dense component, such as ferritin, so as to be available by observation. The Sig moiety could also include a radioactive isotope component, such as radioactive cobalt, making the resulting nucleotide observable by radiation detecting means. The Sig moiety could also include a hapten component or per se be capable of complexing with an antibody specific thereto. Most usefully, the Sig moiety is a polysaccharide or



oligosaccharide or monosaccharide, which is capable of complexing with or being attached to a sugar or polysaccharide binding protein, such as a lectin, e.g. Concanavilin A. The Sig component or moiety of the special nucleotides in accordance with this invention could also include a chemiluminescent component.

As indicated in accordance with the practices of this invention, the Sig component could comprise any chemical moiety which is attachable either directly or through a chemical linkage or linker arm to the nucleotide, such as the base B component therein, or the sugar S component therein, or the phosphoric acid P component thereof.

The Sig component of the nucleotides in accordance with this invention and the nucleotides and polynucleotides incorporating the nucleotides of this invention containing the Sig component are equivalent to and useful for the same purposes as the nucleotides described in the above-identified U.S. patent application Serial No. 255,223. More specifically, the chemical moiety A described in U.S. patent application Serial No. 255,223 is functionally the equivalent of the Sig component or chemical moiety of the special nucleotides of this invention. Accordingly, the Sig component or chemical moiety of nucleotides of this invention can be directly covalently attached to the P, S or B moieties or attached thereto via a chemical linkage or linkage arm as described in U.S. patent application Ser. No. 255,223, as indicated by the dotted line connecting B and A of the nucleotides of U.S. Serial No. 255,223. The various linker arms or linkages identified in U.S. Ser. No. 255,223 are applicable to and useful in the preparation of the special nucleotides of this invention.

Even further embodiments of the instant nucleotides and compositions are later described in the specification, on page 103, first full paragraph; and on page 103, last paragraph, continuing through page 106, first paragraph.

B. In all, there are no fewer than nine (9) instances where the Sig moiety component is described in the specification as being attached to the phosphate moiety P, the sugar moiety S and/or the base moiety B! These nine separate and distinct instances include the following:

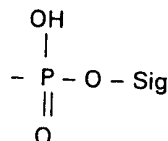
<u>Specification</u>	<u>Description</u>
page 90, last paragraph	... and a signalling chemical moiety Sig covalently attached thereto, either to the P, S or B moiety.
page 93, first paragraph	... include a chemical moiety Sig covalently attached to the P, S and/or B moieties.
page 96, first paragraph	... by having covalently attached thereto, to the P moiety and/or the S moiety and/or the B moiety, a chemical moiety Sig.

- page 98, first paragraph . . . the Sig component or chemical moiety of nucleotides of this invention can be directly covalently attached to the P, S or B moieties or attached thereto via a chemical linkage or linkage arm . . .
- page 103, first full paragraph . . . and the signalling or self-detecting moiety, Sig, covalently attached to either the P, S or B moieties, as indicated hereinabove, . . .
- page 104, first paragraph . . . nucleotides in accordance with this invention containing the above-described components P, S, B and Sig, . . .
- page 105, first paragraph . . . the nucleotides of this invention include the P, S, B and Sig components wherein the Sig is covalently attached to either the P, S or B moieties
- page 105, second paragraph The moiety Sig attached to the special nucleotides of this invention containing the other moieties or components P, S, B provides a site per se for the attachment thereto, the Sig component, . . .
- page 106, first paragraph . . . the special P, S, B and Sig-containing nucleotides of this invention, . . .

C. In addition to those portions in the specification cited above, Example V describes a method for attaching biotin, one of the embodiments for Sig, to the phosphate moiety of a mononucleotide and an oligonucleotide that are coupled to a protein, poly-L-lysine. Using the procedure in Example V in the specification (page 57), the biotinylated poly-L-lysine is coupled to a terminal oxygen of the phosphate moiety or to a terminal phosphorus. These reaction schemes are set forth in Figure 1 on page 374 in Halloran and Parker, *J. Immunol.*, 96:373 (1966) cited in Example V, page 57 in the specification (a copy of Halloran's publication also having been attached hereto as Exhibit 1).

D. In my opinion, the originally filed claims are a telling piece of evidence with respect to using any of the embodiments of Sig for either the base, sugar or phosphate moieties. Here, one need only look at original claim 143 that recites:

A nucleotide having the general formula P-S-B wherein P is the phosphoric acid moiety, S the sugar or monosaccharide moiety and B the base moiety, said nucleotide having covalently attached to the P or S or B moiety a chemical moiety Sig, said Sig chemical moiety when attached to the P moiety is attached thereto via the chemical linkage,



and when Sig is attached to the S moiety, the S moiety is a ribose group, said chemical moiety Sig when attached to said P, S or B being capable of signalling itself or makes itself self-detecting or its presence known.

It is clear from the language of original claim 143 that Sig could be attached to the phosphate (or phosphoric acid), sugar and base moieties in accordance with this invention.

E. The chemical reactions by which substituents are attached to the oxygen or the phosphorus atoms of a phosphate or phosphoric acid moiety in a nucleotide (or an oligo- or polynucleotide or other polymer such as a protein) were known in the art prior to the first filing of this application in 1982. Illustrative of the reactions and chemistry known in the art before 1982 are those summarized below.

#### Reactions involving the oxygen

Goody et al., *JACS* 93:6252-6257 (1971) [Exhibit 2] disclose a reaction for adding a diphenyl to the oxygen atoms of nucleoside di- and triphosphates. See, for example, Scheme I on page 6253.

Eckstein et al., *Biochemistry* 14:5225-5232 (1975) [Exhibit 3] disclose guanosine 5'-di- and triphosphate derivatives with modified terminal phosphates in which the following substituents are added to the terminal oxygen: methyl, aminoethyl, acetylaminoethyl and phenyl. See, for example, the discussion under "Synthesis of Analogues" beginning on page 5226, left column, and continuing through page 5228, right column. See also Figure 1 on page 5226.

Armstrong et al., *European Journal of Biochemistry* 70:33-38 (1976) [Exhibit 4] disclose ATP and UTP analogues modified in the phosphate moieties in which a methyl or a phenyl group is attached to a terminal oxygen. See structures I b) and I c) on page 33, right column.

Reactions involving the phosphorus

Miller et al., Biochemistry 18:5134-5143 (1979) [Exhibit 5] disclose a series of dideoxyribonucleoside methylphosphonate analogues in which a methylene group is contained in the internucleoside linkage. See, for example, the discussion under "*Preparation of Dinucleoside Methylphosphonates*" beginning on page 5136, right column, and continuing through page 5137.

Miller et al., Biochemistry 20:1874-1880 (1981) [Exhibit 6] disclose the preparation of oligodeoxyribonucleoside methylphosphonates. See, for example, the discussion under "*Preparation of Oligonucleoside Methylphosphonates*" beginning on page 1875, left column, and continuing through the first four lines of the right column. See also Table I at the top of page 1876.

Beaucage et al., Tetrahedron Letters 22:1859-1862 (1981) [Exhibit 7] disclose deoxynucleoside phosphoramidites depicted in structures Ia-d and IIIa-d on page 1859 and structures Ia, II and IIIa on page 1861.

Miyoshi et al., Nucleic Acids Research 8:5491-5505 (1980) [Exhibit 8] disclose the preparation of three oligonucleotides, i.e., hexadecanucleotides in which di- and trinucleotides are used as incoming 3'-phosphodiester units. See, for example, Figure 1 on page 5493.

Gait et al., Nucleic Acids Research 8:1081-1096 (1980) [Exhibit 9] disclose the preparation of oligodeoxyribonucleotides up to 12 units long using phosphotriesters.

Duckworth et al., Nucleic Acids Research 9:1691-1706 (1981) [Exhibit 10] disclose the preparation of heptadecadeoxyribonucleotides using phosphotriesters.

Ohtsuka et al., Tetrahedron Letters 23:3081-3084 (1982) [Exhibit 11] disclose the synthesis of dodecadeoxynucleotides using phosphotriesters.

Gough et al., Tetrahedron Letters 22:4177-4180 (1981) [Exhibit 12] disclose the construction of oligodeoxyribonucleotides using phosphotriesters.

In addition to the coupling reactions disclosed in Halloran et al. cited on page 57 in the instant specification (copy attached as Exhibit 1), other procedures were known in the art prior to 1982 for coupling nucleic acid sequences to other biological polymers, including protein and polysaccharides. Among the coupling reactions known before 1982 are those listed below.

Reactions for coupling nucleic acids to other polymers (e.g., proteins, polysaccharides)

Brutlag et al., Biochemistry 8:3214-3218 (1969) [Exhibit 13] disclose cross-linking deoxyribonucleic acid to histone in nucleohistone using formaldehyde.

Manning et al., Chromosoma 53:107-117 (1975) [Exhibit 14] disclose the attachment of biotin to *Drosophila* rRNA via a cytochrome c bridge.

Politz et al., Biochemistry 20:372-378 (1981) [Exhibit 15] disclose the cross-linking of RNA to protein in *Escherichia coli* 30S ribosomal subunits using a heterobifunctional cross-linking reagent.

Cramer et al., Chemische Berichte 92:384-391 (1959) [Exhibit 16] disclose the attachment of polynucleotide sequences to polysaccharides in which 20 units of the latter was described as preferred.

ADEQUATE DESCRIPTION

10. A. It is my opinion that the above-cited portions in the specification adequately describe the presently claimed invention, including particularly those embodiments for the Sig component set forth in the claims. My opinion extends to those embodiments cited by the Examiner in the June 20, 1996 Office (page 3, first paragraph), specifically those where Sig is a moiety containing at least three carbon atoms (claims 280, 312, 340, 376, 408 and 436); Sig includes a glycosidic linkage moiety (claims 287, 319, 347, 380, 412 and 443); Sig is selected from biotin and iminobiotin (claims 288, 321, 349, 381, 413 and 449); Sig comprises ferritin (claims 289, 322, 350, 382 and 414); and Sig is selected from an antigen,

a hapten and an antibody (claims 288, 321, 349, 381, 413 and 449). It is also my opinion that the specification reasonably conveys the description that Sig may be any of the foregoing when attached to the phosphate moiety in the presently claimed nucleotides and other composition claims comprising the nucleotides because of numerous instances (nine in all!) where Sig is described as being attached to the phosphate moiety P, the sugar moiety S and/or the base moiety B. Original claim 143 is particularly significant, in my opinion, because the language specifically recites "said nucleotide having covalently attached to the P or S or B moiety a chemical moiety Sig . . .". The fact that dependent claims for the various embodiments of Sig were not included with the originally filed claims directed to the phosphate modified nucleotides (claim 141) does not in any way detract from my own conviction and opinion that the support and description for such claims would have been clearly and reasonably conveyed by reading the specification, as described in the portions cited above. It is very clear in my opinion that the specification discloses that the embodiments of Sig are to be applied - without limitation - in the disruptive and semi-disruptive positions of all three moieties recited in the independent claims, i.e., the base, sugar and phosphate moieties.

B. To elaborate further, the claimed products encompassing the various embodiments for Sig being attached to the phosphate moiety are clearly supported by the specification, particularly because all such embodiments for Sig are described as functionally equivalent for purposes of the present invention which is directed to disruptive and semi-disruptive modifications of nucleotides involving the phosphate, sugar and base moieties. The fact that one description of the phosphate-modified nucleotides is found in a paragraph that opens with "By way of summary" is of no import for at least three substantial reasons. First, as discussed above in Paragraph 9B above, there are at least nine separate instances in the specification where the attachment of Sig to any or all of the phosphate, sugar and base moieties is disclosed. The specification reasonably conveys, therefore, that the coinventors were in possession of the instantly claimed embodiments for Sig in the phosphate-modified nucleotides and compositions at the time this application was originally filed in 1982. Second, the paragraph beginning with "By way of summary" itself specifically discloses that "[t]he nucleotides are then modified in accordance with the practices of this invention by having covalently attached thereto, to the P moiety and/or the S moiety and/or the B moiety, a chemical moiety Sig." That recitation also reasonably conveys that the coinventors were in

possession of the instantly claimed subject matter at the time the application was first filed in 1982 because the specification clearly informs the reader that Sig can be attached to any of the three moieties in the nucleotide - and even to more than one moiety at the same time. Third, in the subsequent three paragraphs (page 96, last paragraph, through page 98, first paragraph) that describe embodiments for Sig, at least two instances occur where Sig is described as being attached to the phosphate, sugar or base moieties of the nucleotide:

. . . the Sig component could comprise any chemical moiety which is attachable either directly or through a chemical linkage or linker arm to the nucleotide, such as the base B component therein, or the sugar S component therein, or the phosphoric acid P component thereof.

[page 97, first full paragraph]

. . . the Sig component or chemical moiety of nucleotides of this invention can be directly covalently attached to the P, S or B moieties or attached thereto via a chemical linkage or linkage arm . . .

[page 98, first paragraph]

One could reasonably conclude from reading the three paragraphs describing the embodiments for Sig that each are applicable to not only the base moiety, but the phosphate moiety (and the sugar moiety) as well.

C. All of the foregoing reasons supports my conclusion that the specification reasonably conveys that the coinventors were in possession of the subject matter now being claimed.

D. As explained above in Paragraphs 9C and 9D, numerous reactions were known in the art for modifying the phosphate moiety of a nucleotide. It is my opinion that the specification as filed originally in 1982 reasonably conveys that the coinventors were in possession of the subject matter now being claimed and that that impression would not and does not require recitation of the litany of "phosphate-Sig" reactions indicated by the Examiner in the June 25, 1996 and June 20, 1997 Office Actions. Phosphate-Sig reactions were known in the art and an explicit description of such known reactions would not have been necessary to convey the impression that the coinventors were in possession of the subject matter now being claimed.

ENABLEMENT

11. A. It is also my opinion that the specification provides an enabling disclosure for all of the pending claims in this application. As discussed above in Paragraph 9C, Example V in the specification (page 57) provides a means for labeling the oxygen or the phosphorus of a nucleotide. As also noted above in Paragraph 9D, the chemistry and reactions for attaching substituents to the oxygen or phosphorus atoms in a nucleotidyl phosphate or phosphoric acid moiety were already known in the art at the time the initial application was filed in 1982. Although listed above after Paragraph 9D, the known chemistry and reactions are listed below for the sake of completeness.

<u>Chemistry/Reaction</u>	<u>Citation</u>	<u>Description</u>
oxygen	Goody et al. (1971) [Exhibit 2]	diphenyl addition to oxygen of nucleoside di- & triphosphates
oxygen	Eckstein (1975) [Exhibit 3]	methyl, aminoethyl, acetylaminoethyl & phenyl added to GDP & GTP
oxygen	Armstrong et al. (1976) [Exhibit 4]	methyl & phenyl attached to ATP & UTP analogs
phosphorus	Miller et al. (1979) [Exhibit 5]	methylene addition to prepare dideoxyribo-nucleoside methylphosphonates
phosphorus	Miller et al. (1981) [Exhibit 6]	methylene addition to prepare dideoxyribo-nucleoside methylphosphonates
phosphorus	Beaucage (1981) [Exhibit 7]	preparation of deoxynucleoside phosphoramidites
phosphorus	Miyoshi et al. (1980) [Exhibit 8]	oligonucleotide synthesis using phosphotriesters
phosphorus	Gait et al. (1980) [Exhibit 9]	oligodeoxynucleotide preparation using phosphotriesters



phosphorus	Duckworth et al. (1981) [Exhibit 10]	heptadecadeoxyribonucleotide preparation using phosphotriesters
phosphorus	Ohtsuka et al. (1982) [Exhibit 11]	dodecadeoxynucleotide preparation using phosphotriesters
phosphorus	Gough et al. (1981) [Exhibit 12]	oligodeoxyribonucleotide preparation using phosphotriesters
coupling	Halloran et al. (1966) [Exhibit 1]	coupling biotinylated poly-L-lysine to mono- and oligonucleotides
coupling	Brutlag et al. (1969) [Exhibit 13]	DNA to histone
coupling	Manning et al. (1975) [Exhibit 14]	biotin to rRNA using cytochrome c bridge
coupling	Politz et al. (1981) [Exhibit 15]	RNA to ribosomal protein
coupling	Cramer (1959) [Exhibit 16]	polynucleotide sequences to polysaccharides

B. It is my opinion that the subject matter now being claimed in this application, claims 278-453, could have been practiced in 1982 - with minimal experimentation and not with excessive experimentation - from a reading of the specification, particularly Example V, and further in light of the chemistry and reactions known at that time. The known chemistry and reactions are illustrated by the scientific publications cited in this Declaration above (Exhibits 1-16).

Lastly, although not an issue of enablement or adequate description, I should point out that none of the publications submitted in this Declaration (Exhibits 1-16) disclose or suggest the instantly claimed invention in which a Sig moiety is attached to the phosphate moiety of a nucleotide and which is capable of non-radioactive detection when so attached and further, is capable of incorporation into other compositions, including an oligo- or polynucleotide.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false

Dean L. Engelhardt et al.

Serial No.: 08/479/997

Filed: June 7, 1995

Page 19 (Declaration of Dr. Dean L. Engelhardt in Support of Adequate Description  
and Enablement)

statements and the like so made are punishable by fine or imprisonment, or both,  
under Section 1001 of Title 18 of the United States Code, and that any such willful  
false statements may jeopardize the validity of the application or any patent issued  
thereon.

Nov. 24, 1997

Date

Dean L. Engelhardt  
Dean L. Engelhardt

\* \* \* \* \*

## THE PREPARATION OF NUCLEOTIDE-PROTEIN CONJUGATES: CARBODIIMIDES AS COUPLING AGENTS

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*From the Immunology Division, Department of Medicine, Washington University School of Medicine,  
St. Louis, Missouri*

Received for publication August 2, 1965

For some time it has been apparent that antibodies might be useful in the study of the fine structure of RNA and DNA. A major stumbling block has been the unavailability of a method which would render polynucleotides antigenic yet largely preserve their structural integrity. In approaching the problem of covalently conjugating polynucleotides to proteins as a means of stimulating antibody formation, we sought a procedure which would employ terminal nucleotide PO<sub>4</sub> or OH groups for coupling. Two ways in which the terminal PO<sub>4</sub> groups might be coupled covalently to protein would involve formation of a phosphodiester bond with protein seryl and threonyl residues or an N-P bond with protein ε-amino groups (Fig. 1, reactions 1 and 2 respectively). On the other hand, the OH group of a terminal sugar residue could react with protein carboxyl groups forming an ester (Fig. 1, reaction 3). Among possible coupling agents, the water soluble carbodiimides seemed especially attractive because they are known to promote the formation of all three types of bonds (1). Evidence will be presented in this communication which indicates that nucleotides do couple to proteins and polylysine under very mild conditions, in the presence of carbodiimides. Investigation strongly implicates formation of N-P bonds as the principle type of linkage. In the accompanying article it will be shown that conjugates of proteins with mononucleotides, oligonucleotides and DNA elicit the formation of antibodies with nucleotide specificity (2). A brief resume of this work has been reported earlier (3).

<sup>1</sup> Postdoctoral Fellow of the United States Public Health Service, Grant 2 T1-AI-219.

<sup>2</sup> Recipient of a Research Career Development Award from the United States Public Health Service.

### MATERIAL AND METHODS

1-Ethyl-3-diisopropylaminocarbodiimide·HCl (EDC)<sup>3</sup> was obtained from the Ott Chemical Company, Muskegon, Michigan. 1-3-Dicyclohexylcarbodiimide (DCC) and 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)-carbodiimide metho-*p*-toluenesulfonate (CMC) were obtained from the Aldrich Chemical Company, Milwaukee, Wisconsin. *N*-ethyl-5-phenylisoxazolium-3'-sulfonate, various mononucleotides, purified proteins, snake venom phosphodiesterase and calf intestine phosphomonoesterase, calf thymus and salmon sperm DNA were obtained from the Sigma Chemical Company, St. Louis, Missouri. Puromycin was a gift of Dr. Lillian Recant, Washington University School of Medicine. Polylysine·HBr was a product of the Pilot Chemical Company, Watertown, Massachusetts (MW 70,000-80,000).

3'-O-Acetyl thymidylic acid was synthesized according to the procedure of Gilham and Khorana (4). Tetrathymidylic acid was prepared and purified by the method of Khorana and Vizsolyi (5). The *N*-butylamine phosphoroamidate of adenylic acid was prepared as described in reference (6).

*Coupling of mono- and oligonucleotides to proteins.* The following will serve as an example of the procedure used in the coupling of mono-

<sup>3</sup> The following abbreviations are used throughout this article: 1-ethyl-3-diisopropylaminocarbodiimide·HCl, EDC; 1-3-dicyclohexylcarbodiimide, DCC; 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)-carbodiimide metho-*p*-toluenesulfonate, CMC; bovine γ-globulin, B<sub>7</sub>G; *N*-ethyl-5-phenylisoxazolium-3'-sulfonate, reagent K; human serum albumin, HSA; thymidylic acid, T5'-PO<sub>4</sub>; 3'-O-acetyl thymidylic acid, 3'-O-Ac-T5'-PO<sub>4</sub>; tetrathymidylic acid, (T5)<sub>4</sub>; T5'-PO<sub>4</sub>-DCC-HSA and T5'-PO<sub>4</sub>-CMC-HSA are the protein conjugates of T5'-PO<sub>4</sub> with HSA in the presence of the respective coupling agent.

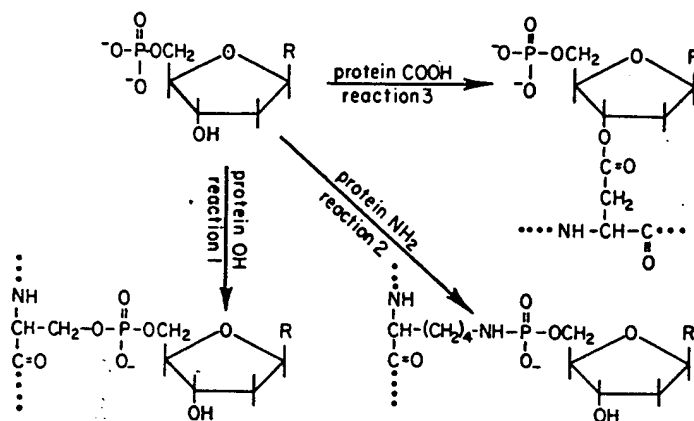


Figure 1. Possible reactions of nucleotides with proteins in the presence of carbodiimides. Very unstable products such as acyl phosphates are not shown.

nucleotides to protein: Human serum albumin (HSA), 25 mg, and 65 mg of thymidylic acid (T5'-PO<sub>4</sub>) were dissolved in 0.5 ml H<sub>2</sub>O. The pH was adjusted to 7.5 and 65 mg EDC were added. The reaction mixture was incubated for 24 hr at room temperature in the dark. The clear solution was dialyzed at 4°C against 0.01 M Tris chloride, pH 7.6, to a constant 267/280 mμ absorbency and phosphorus content (7). Protein concentration was measured by dry weight (8) and the Lowry technique (9).

The same general procedure was used in the coupling reactions involving adenylic acid, Puro-mycin, adenine, adenosine, thymidine and 3'-O-acetyl thymidylic acid to both bovine γ-globulin (BγG) and human serum albumin (HSA).

Coupling with another water soluble carbodi-imide, CMC, was carried out as described for EDC.

Seventy-five milligrams of dicyclohexylcarbodi-imide (DCC) was incubated for 10 min with 60 mg 3'-O-acetylthymidine 5'-PO<sub>4</sub> (3'-O-Ac-T5'-PO<sub>4</sub>) in 1 ml of dry pyridine. The pyridine solution was then added dropwise with rapid stirring to a solution of 25 mg HSA in H<sub>2</sub>O at pH 7.5. After 12 hr the reaction mixture was purified by dialysis as described above.

Attempts to couple T5 to proteins using Woodward's reagent K and tosyl chloride (10) were carried out under the conditions described above (e.g., in aqueous solution with and without preliminary incubation of the mononucleotide with the activating agent in dry pyridine).

Twenty-seven milligrams of tetrathymidylic

acid (T5)<sub>4</sub> were reacted with 4.2 mg HSA and 13 mg EDC in 0.25 ml H<sub>2</sub>O at pH 7.5. The product was dialyzed for 1 week at room temperature and 1 week at 4°C. During the final week of dialysis the absorbency of the protein (T5)<sub>4</sub> solution at 267 mμ remained constant.

**Coupling of DNA to protein.** Highly polymerized salmon sperm DNA was denatured by boiling a 5 mg/ml solution for 10 min and then plunging the solution into a water bath containing crushed ice. Two milliliters of the denatured DNA solution were added to 20 mg of BγG dissolved in 1.5 ml of 0.1 N NaCl. The pH was adjusted to 7.5 and 20 mg EDC were added. The reaction mixture was allowed to incubate for 24 hr at room temperature in the dark and purified by dialysis against 0.01 M Tris-buffered saline. A small amount of precipitate which formed during the incubation was removed by centrifugation at 10,000 rpm for 1 hr. The material was analyzed by electrophoresis employing cellulose acetate and urea starch gel with varying pH conditions. Electrophoresis in the absence of urea was performed on cellulose acetate in 0.05 M barbital, pH 8.6, and in 0.05 M carbonate, pH 10, for 3 hr at room temperature, at 0.4 ma/cm strip. Electrophoresis also was carried out in starch gel containing 7 M urea, 0.05 M formate, pH 3.4, at 125 v for 8 hr. Protein and DNA were localized by Amidoschwartz and Feulgen stains respectively. Controls included the protein alone, EDC-treated protein alone, DNA alone, and mixtures of DNA with untreated and EDC treated protein. The amounts of DNA and protein loaded on the strips in the

TABLE I

*Extent of coupling of nucleosides and nucleotides to proteins and polyaminoacids*

Macromolecule	Nucleotide, Nucleoside or Base	Coupling Agent	Degree of Substitution per Molecule of Carrier <sup>a</sup>	Reactive Groups per Molecule of Carrier
HSA	T5' PO <sub>4</sub>	EDC	23	
HSA <sup>b</sup>	T5' PO <sub>4</sub>	DCC	16	
HSA	T5' PO <sub>4</sub>	CMC	13	
HSA	Adenine	EDC	<1	
HSA	Thymidine	EDC	3	
HSA	Adenosine	EDC	5	
HSA	T5' PO <sub>4</sub>	Tosyl chloride	<0.5	
HSA	T5' PO <sub>4</sub>	Reagent K	<0.5	
B γ G	T5' PO <sub>4</sub>	EDC	28	
B γ G	T5' PO <sub>4</sub>	CMC	17	
Poly-400-lysine <sup>c</sup>	T5' PO <sub>4</sub>	EDC	100	400
Poly-400-lysine	3' O-Ac-T5' PO <sub>4</sub>	EDC	100	400
Hydroxyethyl-poly-400-lysine	T5' PO <sub>4</sub>	EDC	<2	>400
Poly-600-glutamate	T5' PO <sub>4</sub>	EDC	2	600

<sup>a</sup> Conjugates were prepared, purified, and analyzed as described in the text.

<sup>b</sup> A mixture of DCC and T5'-PO<sub>4</sub> in dry pyridine was added dropwise to stirred protein solution (see Material and Methods).

<sup>c</sup> With minor changes in the reaction conditions, i.e., lowering total volume of the reaction mixture, conjugation in the ratio of 200 T5'-PO<sub>4</sub> groups per 400 lysyl residues could be obtained.

controls were comparable to the amounts present in the conjugates.

*Coupling of nucleotides to polyamino acids.* Ten milligrams of polylysine-HBr were dissolved in 0.25 ml H<sub>2</sub>O and 35 mg of T5'-PO<sub>4</sub> were added. The pH was adjusted to 7.5 and 35 mg of EDC were added. The preparation was incubated for 24 hr at room temperature in the dark and then dialyzed as described above. In a similar fashion the reaction of T5'-PO<sub>4</sub> and EDC with polytyrosine and polyglutamic acids was evaluated.

To evaluate further the possible reaction of T5'-PO<sub>4</sub> with hydroxyl groups, hydroxylation of a high molecular weight polylysine with ethylene oxide was carried out.<sup>4</sup> The polylysine was incubated at pH 8 to 9 with a 100-fold molar excess of ethylene oxide (with respect to polymer NH<sub>2</sub>) for 12 hr and purified by dialysis. The ninhydrin reaction of the product was negative, indicating quantitative hydroxyethylation of amino groups. The hydroxyethyl-polylysine was then reacted with T5'-PO<sub>4</sub> at pH 7.5 in the presence of EDC.

Enzymatic digestions of conjugates with snake venom phosphodiesterase and calf intestine phosphomonoesterase were carried out as described in references (12) and (13). Hydroxyl-

amine treatment of conjugates was carried out as described in reference (14).

#### RESULTS

Preliminary experiments conducted with thymidylic acid and human serum albumin in aqueous solution, in the presence of the water soluble carbodiimide 1-ethyl-3-diisopropylamino carbodiimide (EDC), indicated that conjugation of the mononucleotide with protein had taken place. At neutral pH and room temperature a soluble product which contained 23 thymidylate residues/molecule was obtained (Table I). The absorption spectrum of the product as compared with those of the unsubstituted protein and of the protein reacted with EDC alone is shown in Figure 2. The difference spectrum corresponded very closely to that of free thymidylic acid in the 250 to 280 mμ region. The number of thymidylate residues on the protein as estimated by the ultraviolet spectrum corresponded closely to the value obtained by quantitative phosphorus analysis.

Subsequent studies indicated that the conjugation reaction could be extended to other mononucleotides and other proteins using several different carbodiimides (Table I). Even the water insoluble carbodiimide, DCC, could act

<sup>4</sup> For a description of the reaction of ethylene oxide with proteins, see reference (11).

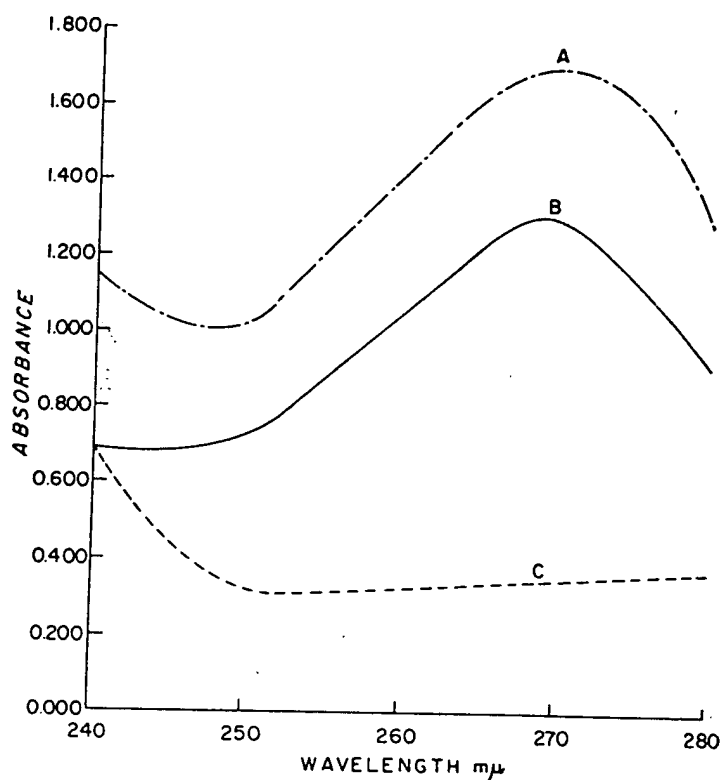


Figure 2. Absorption spectra of HSA and EDC-treated HSA (Curve C), and T5'-PO<sub>4</sub>-EDC-HSA (curve A), each at concentrations of 0.7 mg/ml. The third curve (B) is a difference spectrum in which the protein contribution to the absorbance of T5'-PO<sub>4</sub>-EDC-HSA has been subtracted.

as a coupling agent provided the nucleotide was incubated with DCC in dry pyridine before addition to the protein in aqueous solution (Table I). By contrast, Woodward's reagent K and tosyl chloride, two agents known to promote phosphodiester bond formation under anhydrous conditions, did not result in significant coupling.

In investigating the mechanism of the carbodiimide-induced conjugation of the nucleotide with protein, any substantial participation by the 3' OH group in an ester bond (Fig. 1, reaction 3) could be readily excluded: *a*) 3'-O-acetyl thymidylic acid, which lacks a reactive group at the 3' position, coupled as readily with protein and polylysine as thymidylic acid (Table I); *b*) when the conjugate of thymidylic acid with human serum albumin was subjected to alkaline hydrolysis at 37° in 0.1 M NaOH for various periods of time up to 1 hr and treated with hydroxylamine under conditions which cleave ester bonds (14), 85% or more of the phosphorous remained bound to protein; *c*)

thymidylic acid coupled very sluggishly with polyglutamic acid under the usual reaction conditions (Table I); *d*) while calf intestine phosphomonoesterase cleaved a portion of the phosphorous from protein (see below), thymidine also was removed. This indicated that cleavage was not taking place at a free 5'-PO<sub>4</sub> group.

In a similar manner it could be estimated that relatively few if any of the thymidylate-protein bonds were of the phosphodiester type (Fig. 1, reaction 1): *a*) thymidylate residues were not cleaved to a significant degree from thymidylate-HSA conjugates by snake venom phosphodiesterase; *b*) under the coupling conditions used for proteins, thymidylate acid failed to react appreciably with the hydroxyethylated derivative of polylysine (see Materials and Methods).

The results described above suggested that neither ester nor phosphodiester bonds could account for the majority of the protein-bound thymidylate, and focused attention on the possibility of an N-P bond as the predominant means

of combination (Fig. 1, reaction 2). In accord with this possibility it could be demonstrated that thymidylate readily reacted with polylysine producing a product with as many as 50% of  $\epsilon$ -amino groups of polylysine substituted with thymidylate.<sup>5</sup> Further evidence that N—P bonds can be formed in aqueous solution was obtained by paper chromatography of reaction mixtures containing butylamine, thymidylic acid and various carbodiimides.

On treatment of T5'-PO<sub>4</sub>-HSA conjugates with calf intestine phosphomonoesterase at pH 9.5, thymidine and PO<sub>4</sub> were liberated from the protein, as judged by changes in phosphorous content and the ultraviolet spectrum after dialysis (Table II). Since the enzyme had not been subjected to rigorous purification, the presence of phosphoamidase activity could well account for this result. In accord with this possibility it was found that phosphoamide bonds on T5'-PO<sub>4</sub>-polylysine and the *N*-butylamine phosphoamide of adenylate were cleaved by the enzyme.

While it was evident that mononucleotides could be coupled to proteins by means of carbodiimides, the applicability of the method to high molecular weight polymers remained to be established. Studies with a synthetically prepared tetramer of thymidylate (T5)<sub>4</sub> were encouraging; under the usual coupling conditions a conjugate of (T5)<sub>4</sub> with HSA was obtained which contained at least 50 (T5)<sub>4</sub> residues per molecule of protein. The method also appeared to be applicable to much larger units. Denatured salmon serum DNA (molecular weight 10 million) was reacted with BzG in the presence of EDC. The soluble product obtained was subjected to electrophoresis under a variety of conditions including 7 M urea in starch gel at pH 3.9. No condition was found which led to dissociation of the DNA and protein moieties. By contrast, with mock conjugates between native or EDC-treated protein and

<sup>5</sup> Thymidylic acid also is capable of reacting with carbodiimides which contain secondary and tertiary amino groups. This reaction may assume some importance in protein conjugations using EDC or CMC. Judging from the results of Khorana and his colleagues with various amines, EDC should be capable of reacting directly with protein forming a 1-ethyl-3-diisopropylamino-guanidine substituent on the alkyl side chain of lysyl residues (1). It is possible that a portion of the thymidylate groups become bound to protein by coupling to ethyldiisopropylamino-guanidine.

TABLE II  
*Digestion of HSA-T5' PO<sub>4</sub>-EDC and poly-L-400-T5' PO<sub>4</sub>-EDC with phosphomonoesterase (calf intestine)<sup>a</sup>*

	Enzyme	$\mu$ g P/mg Polylysine or Protein	O.D. at 267 $m\mu$ /mg Polylysine or Protein <sup>b</sup>
Poly-L-400-T5' PO <sub>4</sub> -EDC	0	120	18.150
Poly-L-400-T5' PO <sub>4</sub> -EDC	+	40	6.400
HSA-T5' PO <sub>4</sub> - EDC	0	10	2.400
HSA-T5' PO <sub>4</sub> - EDC	+	6.5	.960

<sup>a</sup> Digestion was carried out on duplicate samples as described in Reference (13) followed by prolonged dialysis against 0.15 M saline, 0.001 N Tris chloride, pH 7.5. Control undigested samples were handled identically with the omission of enzyme.

<sup>b</sup> Corrected for protein contribution at 267  $m\mu$ .

DNA, the protein and DNA could be readily separated. We would infer from these results that a stable linkage was formed between protein and DNA molecules in the presence of EDC.

Investigation of the reaction of adenosine and adenine with protein indicated that the 6-amino group of the base reacts very sluggishly, if at all, with proteins under the usual coupling conditions. The relatively slight degree of conjugation observed with adenosine (Table I) may be due to bonds involving the primary hydroxyl group at the 5' position. Thymidine displayed a reactivity of similar magnitude.

#### DISCUSSION

From the results described above it is evident that carbodiimides afford a means of conjugating nucleotides and oligonucleotides to proteins. Evidence has been presented which indicates that the bulk of the reaction takes place with protein amino groups. It would also appear that denatured DNA forms a stable bond with proteins under similar conditions. However, the nature of the bond here is not established. It is possible that a portion of the binding involves functional groups on purine and pyrimidine bases rather than terminal phosphate groups.

The natural occurrence of N—P—O bonds in  $\alpha$ -casein has been reported by Perlmann (15, 16).

On the basis of results of enzymatic digestion, she inferred that 40% of the total phosphorus was bound in an N—P—O linkage, 20% as pyrophosphate and 40% as a phosphomonoester. She found that complete removal of the phosphorus was accompanied by disintegration of the protein into smaller units.

In the past several years, several methods have been described for combining purine and pyrimidine bases or nucleosides with protein in order to render these materials antigenic. Earlier studies by Butler *et al.* and by Tanenbaum and Beiser employed trichloromethyl purines and pyrimidines for conjugation (17, 18). While our own studies were in progress, Erlanger and Beiser described a reaction involving the vicinal hydroxyl groups on the sugar moiety of ribonucleotides (19). The ribonucleotide was oxidized with sodium periodate, coupled to protein, and the linkage stabilized by reduction. This reaction converted the ribose five-membered ring to a six-membered ring containing a nitrogen derived from the protein. Another recent approach has been that of Sela *et al.* which involves the conversion of mononucleotides or nucleosides to nucleoside-5'-carboxylic acids. The nucleoside-5' carboxylic acid is then coupled to polyamino acids containing free  $\epsilon$ -amino groups (20).

In the method of conjugation described in this article, mononucleotides, oligonucleotides, and DNA are coupled directly to protein. By contrast, the methods described in the preceding paragraph require some form of preliminary chemical modification of the mononucleotide, nucleoside or base. The applicability of these methods to coupling larger units (oligo- and polynucleotides) to proteins or polyamino acids has not been established. We believe, therefore, that the general method described herein has certain advantages over previously described procedures.

There are a variety of other phosphorus-containing compounds of biologic interest which might be linked to proteins in the presence of carbodiimides. Efforts to date to promote the formation of covalent conjugates between flavin mononucleotide and protein with EDC have not been conclusive, however. Clearly, more work is needed before the applicability of the coupling procedure to non-nucleotide monophosphates is established.

In the subsequent article we will describe studies on the antibody specificity of antisera obtained after immunization with these conjugates.

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from  $-0.3$  to  $0.4 \text{ e } \text{\AA}^{-3}$  the bromide ion. The final conventional  $R$  factor is  $2.4\%$ .<sup>21</sup>

The structure of  $11 \cdot \text{HBr}$  was solved and refined in an analogous manner. Its final difference Fourier has no features greater than  $0.2 \text{ e } \text{\AA}^{-3}$  in magnitude. The final  $R$  factor is  $2.3\%$ .<sup>21</sup>

The absolute configurations of  $3 \cdot \text{HBr}$  and  $11 \cdot \text{HBr}$  were established by comparison of the observed and calculated structure factors of the significant Bijvoet pairs of reflections<sup>22</sup> for each salt. For  $3 \cdot \text{HBr}$  there were 24 pairs of reflections for which

$F_o(hkl)$  and  $F_o(\bar{h}\bar{k}\bar{l})$  were both greater than  $30 \text{ e}$  and  $|\Delta F_o| = |F_o(hkl) - F_o(\bar{h}\bar{k}\bar{l})|$  was greater than  $1.6 \text{ e}$ . The corresponding differences in the calculated structure factors,  $\Delta F_c$ , agreed in sign with their respective  $\Delta F_o$  for all 24 pairs. For  $11 \cdot \text{HBr}$ , the signs of all but one of the  $\Delta F_c$  agreed with the signs of the corresponding  $\Delta F_o$  for the 48 pairs of reflections with  $F_o$  greater than  $15 \text{ e}$  and  $\Delta F_o$  greater than  $1.9 \text{ e}$ .

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(21) Listings of structure factors, coordinates, and thermal parameters for  $3 \cdot \text{HBr}$  and  $11 \cdot \text{HBr}$  will appear following these pages in the microfilm edition of this volume of the Journal. Single copies may be obtained from the Reprint Department, ACS Publications, 1155 Sixteenth St., N.W., Washington, D. C. 20036. Remit check or money order for \$3.00 for photocopy or \$2.00 for microfiche.

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## Thiophosphate Analogs of Nucleoside Di- and Triphosphates

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*Contribution from the Max-Planck-Institut für experimentelle Medizin, Chemische Abteilung, Göttingen, Germany. Received March 1, 1971*

**Abstract:** The chemical synthesis of thiophosphate analogs of nucleoside di- and triphosphates bearing a sulfur at the terminal phosphorus atom by the use of *S*-2-carbamoyl ethyl thiophosphate is described. They can readily be oxidized to their disulfides and are only slowly degraded by alkaline phosphatase. Their reactivity with 5,5'-dithiobis(2-nitrobenzoic acid) was investigated and compared with that of other nucleoside thiophosphates.

Nucleotide analogs modified by replacement of an oxygen of the phosphate group by sulfur have recently been described, and have shown interesting behavior with enzymes involved with nucleic acid metabolism.<sup>1</sup> Analogs of nucleotide anhydrides bearing a sulfur at the  $\alpha$  phosphorus atom have also been prepared, and the triphosphates have been used as substrates for DNA-dependent RNA polymerase to obtain polynucleotides containing a thiophosphate backbone.<sup>2</sup> Adenosine 5'-*O*-(1-thiotriphosphate) is also a substrate for C-C-A pyrophosphorylase, and using this enzyme adenosine 5'-*O*-phosphorothioate can be incorporated into tRNA<sup>Phc</sup>.<sup>3</sup> The compound is, however, a competitive inhibitor for phenylalanyl-tRNA synthetase. These examples illustrate the usefulness of the 1-thiophosphate analogs, but a difficulty arises in their use in kinetic studies with enzymes, since the compounds exist as diastereoisomeric pairs due to the asymmetry at the  $\alpha$  phosphorus atom. It is possible for the two isomers to have quite different behavior toward a particular enzyme, and this can present difficulties in interpretation of kinetic results. In the extreme, it is possible that one diastereoisomer is a substrate, while the other is an inhibitor. An analogy for this is provided by the *O*-(*p*-nitrophenyl) ester of thymidine 5'-*O*-thiophosphate, one diastereoisomer of which is a substrate for snake venom phosphodiesterase, while the other is an inhibitor.<sup>4</sup> When the isomers

can be separated, as is the case with uridine 2',3',*O*,*O*-cyclophosphorothioate,<sup>5</sup> the isomerism can be useful to help define the stereochemistry of enzymatic processes.<sup>6</sup> However, for di- and triphosphates, which are difficult to crystallize, separation of the isomers is likely to be impossible. It was therefore thought of interest to attempt the preparation of di- and triphosphate analogs bearing sulfur on the terminal phosphorus atom, since these compounds would have no asymmetry at this site. The compounds were also of interest as potential affinity labels for enzymes containing thiol or disulfide groups in the region of their active site, because of the possibility of disulfide formation with the thiophosphate residue.

Preparation of nucleoside 5'-di- and -triphosphates is normally achieved by activation of a nucleoside 5'-phosphate followed by attack on the activated product using either orthophosphate or pyrophosphate ion. Because of the possibility of attack of thiophosphate ion on such a system *via* either sulfur or oxygen, two possible products can be envisaged from this type of reaction, one having the sulfur on the terminal phosphorus, and the other having a sulfur bridge between the two phosphorus atoms. The first activation method employed was that using *N,N'*-carbonyldiimidazole.<sup>7</sup> The reaction of adenosine 5'-phosphorimidazolates with thiophosphate ion under the reported conditions gave no diphosphate-like product, even after allowing the reaction mixture to stand for a much longer time than is

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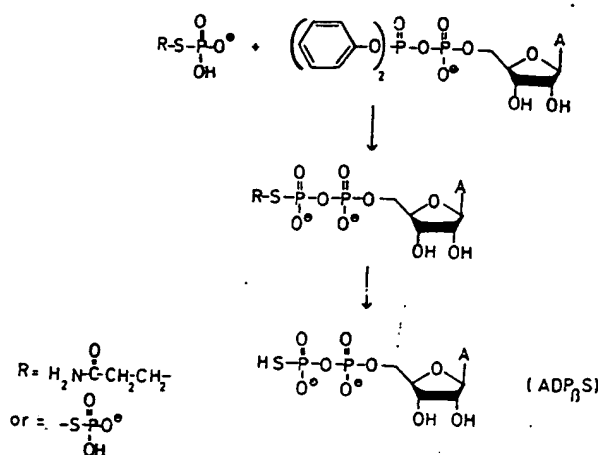
necessary with the orthophosphate ion. After 3 days, the only new product was  $P^1, P^2$ -diadenosine 5'-pyrophosphate, which was detected by electrophoresis and tlc. This is a recognized by-product in the preparation of nucleotide anhydrides.<sup>7</sup>

Since thiophosphate ion showed low reactivity toward the phosphorimidazolite, another procedure was attempted using a more reactive leaving group for activation of the mononucleotide. The procedure used was that described by Michelson in which  $P^1$ -diphenyl  $P^2$ -adenosine 5'-pyrophosphate is allowed to react with orthophosphate.<sup>8</sup> With thiophosphate, no sulfur-containing product was detected after the standard reaction time of 3 hr. After 3 days, small amounts of two sulfur-containing compounds were present with electrophoretic mobilities at pH 7.5 similar to adenosine di- and triphosphates, respectively. The diphosphate-like material was rapidly degraded by alkaline phosphatase to adenosine and was not further studied. The product which behaved like adenosine 5'-triphosphate at pH 7.5 was found to have a phosphorus to adenine ratio of 2:1, and at pH 3.5 had the same electrophoretic mobility as adenosine 5'-diphosphate. In this respect, it was identical with adenosine 3',5'- $O$ , $O$ -diphosphate. Degradation with alkaline phosphatase gave adenosine 3'-thiophosphate, this product then being stable to further degradation.<sup>1</sup> From this evidence, it seems likely that the compound is adenosine 3'- $O$ -thiophosphate 5'-phosphate. An analogy for the formation of this compound is provided by the dismutation reactions of nucleoside 5'-polyphosphates in pyridine, where at least one compound having a 3'-phosphate group is formed, probably by an intramolecular phosphorylation.<sup>9</sup>

Due to the difficulties encountered above, another approach was attempted, which is an extension of the procedure used for the preparation of nucleoside 5'- $O$ -thiophosphates.<sup>1</sup> Adenosine 5'-phosphate was protected by conversion to the  $N^6, 2'-O, 3'-O$ -triacetate,<sup>10</sup> and then allowed to react with triimidazole 1-phosphinesulfide. Successive treatments with acetic acid and ammonia to remove all protecting groups gave a small amount of diphosphate-like product. However, this was shown not to contain sulfur, and was in fact adenosine 5'-diphosphate. It seems likely that the reaction proceeded in the expected fashion, but that under the acidic conditions necessary to remove the second imidazole group, sulfur was also lost.

As noted above, the presence of the  $P=S$  grouping in the thiophosphate ion apparently has an adverse effect on the reactivity of the ion toward an activated adenosine 5'-phosphate derivative. It was therefore decided to protect the sulfur by esterification, in the hope that this might lessen its influence. A requirement for the ester grouping was that it should be removable under reasonably mild conditions. The group chosen for this purpose was that introduced by Cook,<sup>11</sup> the carbamoyl ethyl group (Scheme I). Reaction of  $S$ -2-carbamoyl ethyl thiophosphate with  $P^1$ -diphenyl  $P^2$ -adenosine 5'-pyrophosphate proceeded smoothly, and after removing the protecting group under alkaline

Scheme I



conditions, adenosine 5'- $O$ -(2-thiodiphosphate)(ADP $\beta$ S) was isolated in 35% yield by chromatography on DEAE-cellulose. The compound was identified by its electrophoretic mobility (identical with adenosine 5'-diphosphate at pH 3.5 and 7.5), its different chromatographic behavior compared to adenosine 5'-diphosphate, its nonidentity with adenosine 5'- $O$ -(1-thiodiphosphate), and the development of a white color when a chromatogram was sprayed with a starch-iodine solution. The compound was only very slowly degraded by alkaline phosphatase, about 50% being degraded in 12 hr under conditions where adenosine 5'-diphosphate is degraded in less than 10 min. The compound behaves as a competitive inhibitor for this enzyme ( $K_i = 6.6 \times 10^{-5} M$ ). Evidence that the sulfur is situated on the terminal phosphorus and not between the two phosphorus atoms is provided by the reversible oxidation of the compound to its disulfide using potassium ferricyanide or hydrogen peroxide, and by its reaction with Ellman's reagent (see below). The disulfide was found to be stable to alkaline phosphatase. It could be readily separated from the free thiophosphate by tlc or paper electrophoresis.

Another approach to the preparation of this analog involved protection of the thiophosphate by oxidation to its disulfide with iodine in hydrochloric acid,<sup>12</sup> and use of this ion as the attacking species in a reaction with  $P^1$ -diphenyl  $P^2$ -adenosine 5'-pyrophosphate. After removal of the protecting thiophosphate group by reduction with 2-mercaptoethanol, adenosine 5'- $O$ -(2-thiodiphosphate) was obtained in 25% yield.

For the preparation of adenosine 5'- $O$ -(3-thiotriphosphate) (ATP $\gamma$ S) an extension of the first method described above for the 2-thiodiphosphate was attempted. Adenosine 5'-diphosphate was activated by reaction with diphenyl phosphorodichloridate, and the product (not isolated) was treated with  $S$ -2-carbamoyl ethyl thiophosphate. After removal of the protecting group with 0.2  $N$  sodium hydroxide at 100°, and chromatography on DEAE-cellulose and DEAE-Sephadex, adenosine 5'- $O$ -(3-thiotriphosphate) was obtained in ca. 4% yield. The compound was slightly more stable than the 2-thiodiphosphate to alkaline phosphatase, about 48 hr being needed for 50% hydrolysis under the standard conditions used. Electrophoretically, the compound was identical with adenosine 5'-triphosphate at pH 3.5 and 7.5. It could be distinguished from the

(8) A. M. Michelson, *Biochim. Biophys. Acta*, **91**, 1157 (1964).

(9) J. R. Reiss and J. G. Moffatt, *J. Org. Chem.*, **30**, 3381 (1965).

(10) R. K. Ralph and H. G. Khorana, *J. Amer. Chem. Soc.*, **83**, 2926 (1961).

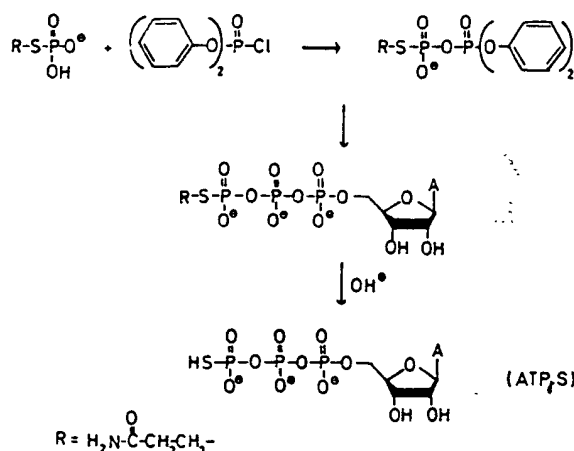
(11) A. F. Cook, *Ibid.*, **92**, 190 (1970).

(12) E. Thilo and E. Schöne, *Z. Anorg. Chem.*, **259**, 226 (1949).

latter by tlc on cellulose impregnated with polyethylenimine (PEI cellulose). Like the 2-thiodiphosphate, the compound could be oxidized with ferricyanide or hydrogen peroxide to give a disulfide which could be reduced back to the starting material with dithiothreitol or 2-mercaptoethanol.

Since this method resulted in a low yield of the desired product, an alternative procedure was investigated (Scheme II). S-2-Carbamoylethyl thiophosphate was

Scheme II



activated by reaction with diphenyl phosphorochloridate, and the product, which was not characterized, was allowed to react with adenosine 5'-diphosphate. Following the same hydrolysis and work-up procedure as before, the 3-thiotriphosphate was isolated in 25% yield after DEAE-cellulose chromatography slightly contaminated with inorganic thiophosphate and adenosine 5'-triphosphate, or in slightly lower yield following DEAE-Sephadex chromatography. The absence of adenosine 5'-triphosphate in this product was best shown by tlc on PEI-cellulose. The 5'-O-(3-thiotriphosphates) of uridine and guanosine were prepared in the same way.

In an attempt to estimate the amounts of sulfur in the two compounds whose preparations have been described, their reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent<sup>13</sup>) was investigated. The extent of reaction with the reagent was estimated from the light absorbance at 412 mμ of the released 2-nitro-5-thiobenzoic acid ion. It was found that with both compounds the extent of reaction was very small, unless a large excess of Ellman's reagent was used. By stepwise addition of the reagent, the theoretical limit of reaction could be approached, but never reached. Clearly, the reaction is reversible, and the position of equilibrium very much favors the existence of the thiophosphates in the reduced state. Table I shows equilibrium constants calculated from the spectrophotometric data for the two analogs, along with those of several other analogs. In their calculation, it was assumed that only mixed disulfide formation between a thiophosphate and 2-nitro-5-thiobenzoic acid was of importance, and that symmetrical disulfide formation was negligible, due to the large excess of Ellman's reagent used. Relative initial rates of reaction under identical conditions are also given (Table I).

(13) G. A. Ellman, *Arch. Biochim. Biophys.*, **82**, 70 (1959).

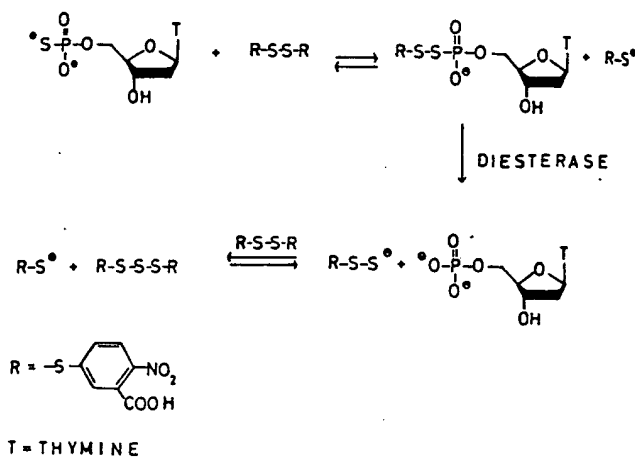
Table I. Reaction of Thiophosphate Analogs with Ellman's Reagent

Compd	Rel initial reaction rate	K <sup>a</sup>
AMPS <sup>b</sup>	1.0	$3.22 \times 10^{-3}$
TMPS <sup>b</sup>	0.88	$3.45 \times 10^{-3}$
ADPβS	0.33	$4.15 \times 10^{-3}$
ATPγS	0.091	$2.32 \times 10^{-3}$
ATPαS <sup>c</sup>	0.081	$5.35 \times 10^{-3}$
3'(2')UMPS <sup>c</sup>	0.41	

<sup>a</sup> K = equilibrium constant for the reaction  $RPSH + R'SSR' \rightleftharpoons RPSSR' + R'SH$ , where R = a nucleoside or nucleotide and R' = 2-nitro-5-thiobenzoic acid. <sup>b</sup> Reference 1.

It is of interest to note that addition of snake venom phosphodiesterase to the mixture of Ellman's reagent and thymidine 5'-O-thiophosphate or adenosine 5'-O-thiophosphate resulted in the release of 2 equiv of colored anion. The most likely reaction path to explain this result is the following (Scheme III).

Scheme III



Presumably the last stage is driven to completion, or nearly so, by the large excess of Ellman's reagent used. This explains the formation of 2 equiv of anion.

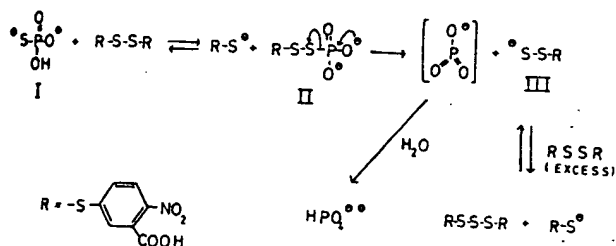
The intermediate disulfide seems a likely substrate for the enzyme, because of its resemblance to a diester of orthophosphoric acid. An alternative reaction scheme, in which the analog is desulfurized by the phosphodiesterase and the resulting hydrogen sulfide reacts with Ellman's reagent liberating 2 equiv of anion, could be ruled out in the case of adenosine 5'-thiophosphate, since reaction of the phosphodiesterase with the analog in the absence of Ellman's reagent resulted in much slower desulfurization.

Addition of snake venom phosphodiesterase to the mixtures of thiodi- or triphosphates with Ellman's reagent caused no further release of color, indicating that in this case the intermediate disulfides are not recognized as substrates.

In the reaction of uridine 3'(2')-O-thiophosphate with Ellman's reagent, the production of 2-nitro-5-thiobenzoic acid did not stop until 2 equiv of the anion were released. Chromatography of the products showed that complete conversion to uridine 2',3'-O-cyclic phosphate had occurred. Presumably this compound was formed by intramolecular attack of the 2'(3')-hydroxyl group on the mixed disulfide of uridine 3'(2')-O-thiophosphate and 2-nitro-5-thiobenzoic acid.

Since it is known that thiophosphate ion can reduce disulfide bonds in certain enzymes,<sup>14</sup> an attempt was made to compare the reactivities of simple thiophosphate ion and the nucleotide analogs toward Ellman's reagent. Reaction of thiophosphate with the reagent resulted in the rapid release of twice as much 2-nitro-5-thiobenzoate as expected, even using a comparatively small excess of the disulfide. Furthermore, from the kinetics of the reaction, it was clear that two separate steps are involved, the first being faster than the second. It seems likely that the following sequence of reactions occurs (Scheme IV).

Scheme IV



This scheme results in the liberation of 2 equiv of anion/equiv of thiophosphate. The suggested mechanism of breakdown is based on the high reactivity of the disulfide III as a leaving group, as already indicated in the formation of uridine 2',3'-O,O-cyclic phosphate from uridine 3'(2')-O-thiophosphate as described above.

Due to the more complex reaction of thiophosphate ion with Ellman's reagent, it was not possible to compare its reactivity with that of the analogs. However, a comparison with cysteine was obtained by mixing cysteine and Ellman's reagent in a 1:1 molar ratio. This resulted in liberation of the maximum theoretical amount of colored anion, indicating a high equilibrium constant for this reaction. This would suggest that cleavage of cysteine disulfide bonds in proteins by the analogs is unlikely, unless it results in some radical change in structure leading to a more stable configuration of the polypeptide chain. However, since the analogs apparently have low reducing power, it follows conversely that their disulfides should have high oxidizing power, i.e., that they would react readily with reactive thiol groups such as that in cysteine.

The interactions of the analogs with several enzyme systems, among them DNA-dependent RNA polymerase and polynucleotide phosphorylase, are under investigation in our laboratory.

## Experimental Section

**General Procedures.** Paper chromatography was performed by the descending method, using Schleicher and Schüll 2043b (washed) paper in system A (ethanol-1 M ammonium acetate, 7:3, v/v) and Whatman 3 MM paper in system B (isobutyric acid-2 N NH<sub>4</sub>OH-0.2 N EDTA, 120:12:2, v/v). Thin-layer chromatography (tlc) using Merck silica gel F<sub>254</sub> plates was carried out using system A. Ion exchange tlcs were performed with MN-Polygram CEL 300 PEI/UV plates (Machery and Nagel, Düren, Germany) eluting with 0.75 M KH<sub>2</sub>PO<sub>4</sub> adjusted to pH 3.4 with concentrated HCl. Schleicher and Schüll 2043b (washed) paper was used for high-voltage electrophoresis in 0.1 M triethylammonium bicarbonate (pH 7.5) or 0.05 M ammonium formate buffer (pH 3.5) at 2200 V for 1.5 hr. Sulfur-containing spots were detected on paper or tlcs

plates using a spray containing sodium azide (1 g), soluble starch (1 g), and iodine (1 g).<sup>15</sup> Using this spray, compounds containing readily oxidizable sulfur appeared as white spots on a blue background. Ultraviolet absorption measurements were made using a Cary Model 14, Zeiss PMQII, or Unicam SP-1800 instrument. <sup>31</sup>P nmr spectra were recorded with a Perkin-Elmer R-10 spectrometer in connection with a Northern Scientific HS-544 Digital Memory oscilloscope, with 30% aqueous phosphoric acid as standard. Chemical shifts are given in  $\delta$  units (parts per million) from standard. Whatman DE-23 or DE-52 cellulose and DEAE-Sephadex A-25 were used for ion exchange chromatography using a linear gradient of triethylammonium bicarbonate.

Degradations with alkaline phosphatase (5–10  $\mu$ l) were carried out using 0.3–1  $\mu$ mol of nucleotide in 0.05–0.15 ml of 0.1 M Tris-HCl buffer (pH 8.0) at 37° for the length of time stated. The products were analyzed by tlcs in system A and electrophoresis at pH 3.5 or 7.5. Non-sulfur-containing nucleotides present as impurities in the preparations were estimated as follows. The uv absorbance of a solution containing the analog (ca. 2 A<sub>260</sub> units in 2.5 ml of 0.1 M Tris-HCl buffer, pH 8.0) and adenosine deaminase (2  $\mu$ l of solution) was measured at 265 m $\mu$ . Alkaline phosphatase (5  $\mu$ l) was added, and the decrease in the absorbance at 265 m $\mu$  was measured. For complete degradation,  $\epsilon_M = -8200$ . Alkaline phosphatase (purity grade I, calf intestine) and adenosine deaminase were purchased from Boehringer and Söhne (Mannheim, Germany), as was snake venom phosphodiesterase.

Phosphate determinations were carried out by the methods of Hurst and Becking<sup>16</sup> or of Chen, *et al.*<sup>17</sup>

Table II. Thin-Layer Chromatography of Nucleotide Anhydrides and Their Thiophosphate Analogs

Compd	$R_f$ values	
	Silica gel	PEI-cellulose
ADP	0.06	0.49
ADP $\beta$ S	0.12	0.27
ADP $\alpha$ S <sup>a</sup>	0.08	0.33
ADP $\beta$ S disulfide	0.49	
ATP	0.03	0.25
ATP $\gamma$ S	0.06	0.10
ATP $\alpha$ S <sup>b,c</sup>	0.03	0.12
ATP $\gamma$ S disulfide	0.37	0.08
GTP	0.0	0.13
GTP $\gamma$ S	0.0	0.05
UTP	0.07	0.50
UTP $\gamma$ S	0.08	0.18
UTP $\gamma$ S disulfide	0.50	

<sup>a</sup> Adenosine 5'-O-(1-thiodiphosphate). <sup>b</sup> Adenosine 5'-O-(1-thiotriphosphate). <sup>c</sup> Reference 2.

**Adenosine 5'-O-(2-Thiodiphosphate) (ADP $\beta$ S).** (1) Adenosine 5'-phosphate (347 mg, free acid, 1 mmol) was added to dry methanol (5 ml) and tri-*n*-octylamine (0.43 ml, 1 mmol), and the mixture was warmed gently until solution was obtained. The solvent was removed under reduced pressure, and the residue was dried by repeated evaporation of 5-ml aliquots of dry DMF. Dry dioxane (7 ml) was added to the residue, followed by dry DMF (1 ml) if solution did not occur easily. Diphenyl phosphorochloridate (0.3 ml) was added to the solution, followed by tri-*n*-butylamine (0.3 ml). The white precipitate obtained at this stage redissolved slowly on stirring. After standing for 3 hr at room temperature, the solvent was removed by evaporation, and dry ether (50 ml) was added to the residue with shaking. The mixture was allowed to stand at 4° for 0.5 hr, and the ether was then removed by decantation. Dry dioxane (5 ml) was added to the residue, and the resulting suspension was evaporated to dryness. A solution of S-2-carbamoyl ethyl thiophosphate (tri-*n*-butylammonium salt, 2 mmol)<sup>18</sup>

(15) D. J. Shire, Göttingen, unpublished results.

(16) R. O. Hurst and G. C. Becking, *Can. J. Biochem. Physiol.* 41, 469 (1963).

(17) P. S. Chen, T. Y. Tosibara, and H. Warner, *Anal. Chem.*, 28, 1756 (1956).

(18) Prepared by passing a solution of the lithium salt (2 mmol)<sup>11</sup> over a column of Merck I ion exchanger (pyridinium form) followed by evaporation and re-solution of the residue in methanol containing tri-*n*-butylamine (2 mmol). After removal of the solvent, the product was dried by repeated evaporation of 5-ml aliquots of dry pyridine.

(14) H. Neumann, R. F. Goldberger, and M. Sela, *J. Biol. Chem.*, 239, 1536 (1964).

in dry pyridine (6 ml) was added and the solution was allowed to stand for 3 hr at room temperature, after which time a precipitate had formed. The pyridine was removed by evaporation under reduced pressure, 0.2 *N* NaOH (80 ml) was added, and the resulting cloudy solution was heated for 10 min at 100°. After cooling, the mixture was neutralized with Merck I ion exchanger (pyridinium form), treated with 2-mercaptoethanol (0.5 ml), filtered, and chromatographed on DEAE-cellulose, using a linear gradient of triethylammonium bicarbonate buffer (pH 7.5) from 0.05 to 0.3 *M*. The product was eluted at about 0.22 *M* (5400 *A*<sub>260</sub> units, 35% yield from adenosine 5'-phosphate). The compound was electrophoretically identical with adenosine 5'-diphosphate, but gave a white color with the sulfur spray; adenosine:phosphorus = 1:2.07;  $\lambda_{\text{max}}^{\text{H}_2\text{O}}$  259 m $\mu$  ( $\epsilon$  15,000). The adenosine 5'-diphosphate content was not more than 1%, as judged by degradation with alkaline phosphatase.

(2) Dipotassium dihydrogen dithiophosphate<sup>18</sup> (302 mg, 1 mmol) was converted to the pyridinium salt by passage of a solution in 50% aqueous methanol down a column of Merck I ion exchanger in the pyridine form. Tri-*n*-butylamine (2 mmol) was added to the effluent, which was then evaporated under reduced pressure. The residue was dried by repeated evaporation of dry pyridine. A solution of this product in dry pyridine (4 ml) was then substituted for *S*-2-carbamoylthiophosphate in the procedure described above, using 0.5 mmol of adenosine 5'-phosphate. After standing for 16 hr at room temperature, the solvent was removed under reduced pressure, and the residue was dissolved in water (20 ml), treated with 2-mercaptoethanol (2 ml), and chromatographed as before on DEAE-cellulose (2000 *A*<sub>260</sub> units, 26% yield from adenosine 5'-phosphate). This product was identical by electrophoresis, tlc, and paper chromatography in system B (*R*<sub>ADP</sub> = 0.75) with that obtained using *S*-2-carbamoylthiophosphate. Its chemical and enzymatic properties were also identical.

Adenosine 5'-*O*-(3-Thiotriphosphate) (ATP $\gamma$ S). *S*-2-Carbamoylthiophosphate (Li<sup>+</sup> salt, 0.5 mmol) was converted to the pyridinium salt by passage over Merck I ion exchanger, and then converted to the mono(tri-*n*-octylammonium) salt by addition of tri-*n*-octylamine (0.22 ml, 0.5 mmol) to a solution in methanol. The solvent was removed under reduced pressure, and the residue was dried by repeated evaporation of dry DMF. The product was then dissolved in dry dioxane (3.5 ml), and diphenyl phosphorochloridate (0.15 ml) followed by tri-*n*-butylamine (0.23 ml) were added. The solution was allowed to stand at room temperature for 2 hr, and then evaporated under reduced pressure. Ether (10 ml) was added to the residue, and after shaking for 1–2 min, petroleum ether (40–60°, 20 ml) was added and the mixture was allowed to stand at 4° for 0.5 hr. The supernatant was decanted, the residue was dissolved in dry dioxane (3 ml), and the solution was concentrated under reduced pressure. A solution of adenosine 5'-diphosphate [mono(tri-*n*-octylammonium), mono(tri-*n*-butylammonium) salt, 0.25 mmol]<sup>19</sup> in dry pyridine (3 ml) was added to the remaining syrup, and the solution was allowed to stand for 2 hr at room temperature. A precipitate formed slowly during this time. The solvent was removed under reduced pressure, 0.2 *N* NaOH (20 ml) was added, and the mixture was heated at 100° for 10 min. After cooling, the cloudy solution was neutralized with Merck I ion exchange resin (pyridinium form), treated with 2-mercaptoethanol (0.5 ml), and chromatographed on DEAE-cellulose using a linear gradient of triethylammonium bicarbonate buffer (pH 7.5, 0.15–0.4 *M*). The product was eluted at about 0.28 *M* (800 *A*<sub>260</sub> units, slightly contaminated with adenosine 5'-diphosphate and thiophosphoric acid). The compound could be obtained pure by chromatography on DEAE-Sephadex using a linear gradient of triethylammonium bicarbonate buffer (pH 7.5,

(19) Prepared by the method of Thilo and Schöne,<sup>12</sup> with the modification that 2 mol of thiophosphate/mol of iodine was used instead of the reported ratio of 1:1. The latter results in a twofold excess of iodine. The identity of the product was confirmed by its elemental analysis (Calcd for K<sub>2</sub>H<sub>2</sub>P<sub>2</sub>O<sub>6</sub>·S<sub>2</sub>: H, 0.7; P, 20.5; S, 21.2. Found: H, 0.84; P, 20.35; S, 21.21) and <sup>31</sup>P nmr spectrum (H<sub>2</sub>O, -1.5). Addition of dithiothreitol caused a shift in the signal to the position of thiophosphoric acid at the appropriate pH (-44).

(20) Adenosine 5'-diphosphate (Na<sup>+</sup> salt, 0.25 mmol) was converted to the pyridinium salt by passage of a solution in 50% aqueous methanol over a column of Merck I (pyridinium form). After removal of solvent, methanol (10 ml), tri-*n*-octylamine (0.25 mmol), and tri-*n*-butylamine (0.25 mmol) were added to the residue. After stirring for 0.5 hr, solution had occurred. The solvent was removed by evaporation, and the residue was dried by repeated evaporation of 5-ml aliquots of dry pyridine.

0.35–0.6 *M*). The product was eluted at about 0.5 *M*. It was electrophoretically identical with adenosine 5'-triphosphate. Non-sulfur-containing contaminants were not detectable by tlc or by degradation with alkaline phosphatase; adenosine:phosphorus = 1:2.94;  $\lambda_{\text{max}}^{\text{H}_2\text{O}}$  259 m $\mu$  ( $\epsilon$  15,000).

Uridine 5'-*O*-(3-Thiotriphosphate) (UTP $\gamma$ S) and Guanosine 5'-*O*-(3-Thiotriphosphate) (GTP $\gamma$ S). These compounds were synthesized in the same way as adenosine 5'-*O*-(3-thiotriphosphate). They were identical on electrophoresis with the corresponding nucleoside 5'-triphosphates but could be readily distinguished from these by PEI-cellulose tlc. They could be oxidized to the disulfides by ferricyanide or H<sub>2</sub>O<sub>2</sub>.

Oxidation of Analogs. Adenosine 5'-*O*-(2-Thiodiphosphate) Disulfide. Oxidation of adenosine 5'-*O*-(2-thiodiphosphate) was carried out with either potassium ferricyanide or hydrogen peroxide.

(1) Potassium ferricyanide solution (0.1 *M*, 100  $\mu$ l) was added to the diphosphate analog (1  $\mu$ mol) dissolved in water (10  $\mu$ l), and after 5 min at room temperature, the whole solution was applied to chromatography paper, and the mixture was separated by electrophoresis at pH 7.5 and 2200 V. At this pH the disulfide has a mobility equal to that of adenosine 5'-phosphate. After elution in methanol-water (1:1, v/v), the product was obtained in ca. 60% yield. It was readily distinguished from the starting material by tlc (Table I). Addition of 2-mercaptoethanol to the disulfide resulted in rapid reduction to the original thiodiphosphate.

(2) For preparation of the disulfide on a larger scale, oxidation with hydrogen peroxide was more convenient. Adenosine 5'-*O*-(2-thiodiphosphate) (6  $\mu$ mol in 0.8 ml of water) was treated with 3% hydrogen peroxide (0.1 ml in a 100-ml round-bottomed flask),<sup>11</sup> and the solution was then immediately evaporated to dryness. Water (1 ml) was added to the residue and again immediately removed by evaporation. This process was repeated three times to remove hydrogen peroxide. As long as the starting material was free of thiophosphoric acid, this procedure resulted in quantitative conversion to the disulfide. Longer contact time with hydrogen peroxide must be avoided to prevent further oxidation of the product.

Adenosine 5'-*O*-(3-Thiotriphosphate) Disulfide. Oxidation of adenosine 5'-*O*-(3-thiotriphosphate) could be achieved in the same way as described for the 2-thiodiphosphate. With potassium ferricyanide under the conditions described above, a 50% yield of disulfide was obtained. For larger scale oxidations, hydrogen peroxide was used. On paper electrophoresis at pH 7.5, the compound had a mobility relative to adenosine 5'-triphosphate of 0.82, and was readily distinguished from the latter by tlc (Table II). Reduction with 2-mercaptoethanol gave the starting material.

Stability of the Analogs against Alkaline Phosphatase. Five *A*<sub>260</sub> units of each of the respective analogs in Tris-HCl buffer (0.1 *M*, pH 8.0, 50  $\mu$ l) were treated with alkaline phosphatase (5  $\mu$ l) at 37°. The mixture was examined by tlc in system A after 2, 30, and 48 hr. Adenosine 5'-*O*-(2-thiodiphosphate) was degraded completely after 48 hr, whereas adenosine 5'-*O*-(3-thiotriphosphate) was less than 50% degraded. Detectable degradation of the 2-thiodiphosphate occurred after 2 hr. Under the same conditions, adenosine 5'-diphosphate was degraded completely after 10 min.

The stability of adenosine 5'-*O*-(2-thiodiphosphate) was high enough to measure inhibition kinetics in the degradation of adenosine 5'-phosphate. Using the assay system described before, the rate of cleavage could be followed by the rate of deamination of adenosine by adenosine deaminase. The inhibitor had a *K*<sub>i</sub> value of  $6.6 \times 10^{-8}$  *M*, compared with a *K*<sub>M</sub> value for adenosine 5'-*O*-phosphate of  $5.95 \times 10^{-8}$  *M*.

The disulfides of the two analogs were stable against alkaline phosphatase under the conditions described above.

Reactions of Thiophosphate Analogs with Ellman's Reagent. About 2 *A*<sub>260</sub> units of the analog (in the case of thymidine 5'-*O*-thiophosphate about 0.7 *A*<sub>260</sub> unit) were dissolved in 2.5 ml of 0.1 *M* Tris-HCl buffer (pH 7.6) in a quartz glass cell (path length 1 cm) and the exact absorbance at 260 or 267 m $\mu$ , respectively, was measured. Ellman's reagent (usually 50  $\mu$ l of a solution in 0.1 *M* Tris-HCl buffer (pH 7.6) saturated at 4°; concentration of the reagent = 11.8 mM) was added, and the increase in absorbance at 412 m $\mu$  was continuously recorded. When the absorbance had reached a constant level (usually within 5 min), more Ellman's reagent (50 or 100  $\mu$ l) was added, and the increase again recorded. From these data, the relative initial rate for each analog was calculated (allowing for slight variations in the amount of analog

(21) It is important to use a flask of this size or larger so that removal of hydrogen peroxide by evaporation is as rapid as possible.

used), as were the equilibrium constants. The latter could be calculated from single measurements, or graphically, after obtaining several points by stepwise addition of Ellman's reagent. The good straight line obtained in the graphical method indicated the validity of the assumption that symmetrical disulfide formation was negligible. Addition of snake venom phosphodiesterase (5  $\mu$ l) to the mixtures of thymidine 5'-O-thiophosphate or adenosine 5'-O-thiophosphate and the reagent resulted in the production of 2 equiv of 2-nitro-5-thiobenzoic acid. When thiophosphoric acid was substituted for an analog in the above procedure, 2 equiv of colored anion were produced in a reaction which involved two steps, as shown by the kinetic data, the first step being significantly faster than the second. Extrapolation of the line for the second step back to zero time indicated that in each step 1 equiv of anion was released.

In the case of uridine 3'(2')-O-thiophosphate, the characteristic pattern of reaching equilibrium after a few minutes was not observed. Instead, the absorbance at 412 m $\mu$  increased steadily until 2 equiv of the anion had been liberated. To investigate the products from this reaction, Ellman's reagent (150  $\mu$ l, 10 mg/2 ml) was added to uridine 3'(2')-O-thiophosphate (4.7  $A_{260}$  units) in 300  $\mu$ l of 0.1 M Tris-HCl buffer (pH 7.4) and the mixture was left overnight. Paper chromatography in system A then showed complete conversion to uridine 2',3'-O,O-cyclic phosphate.

**Reaction of Adenosine 5'-O-Thiophosphate with Snake Venom Phosphodiesterase.** (1) Adenosine 5'-O-thiophosphate (2  $A_{260}$  units) was dissolved in 0.1 M Tris-HCl (pH 7.6), and the light absorbance at 260 m $\mu$  was measured. Adenosine deaminase (3  $\mu$ l), alkaline phosphatase (10  $\mu$ l), and snake venom phosphodiesterase (5  $\mu$ l) were added. The absorbance at 265 m $\mu$  was unchanged after 30 min, indicating that reaction was very slow under these conditions. Addition of Ellman's reagent (25  $\mu$ l of a 11.8 mM solution) resulted in liberation of 2 equiv of 2-nitro-5-thiobenzoic acid/equiv of analog within 10 min.

(2) Adenosine 5'-O-thiophosphate (10  $A_{260}$  units) was incubated overnight at 37° with snake venom phosphodiesterase (10  $\mu$ l) in 100  $\mu$ l of 0.1 M Tris-HCl buffer (pH 7.4). Paper chromatography in system A indicated that no adenosine had been formed. Alkaline phosphatase (10  $\mu$ l) was then added, and the mixture was incubated for a further 2 hr at 37°. Paper chromatography in system A showed the presence of adenosine and adenosine 5'-O-thiophosphate in the ratio 6:4, indicating 60% desulfurization by the phosphodiesterase.

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## Calculation of the Rotational Strengths of Mononucleosides<sup>1</sup>

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**Abstract:** The rotational strengths of the two longer wavelength transitions,  $B_{2U}$  and  $B_{1U}$ , of four mononucleosides (adenosine, guanosine, uridine, and cytidine) as a function of the glycosidic rotational angles have been investigated theoretically. The transition in each base is characterized by transition monopoles; the sugar is treated as a sum of bond polarizabilities. The interaction among these polarizabilities is also considered. Rotational strengths were calculated using three different sets of transition monopoles and many combinations of bond polarizabilities. We conclude that adenosine, uridine, and cytidine may have primarily one conformation, but that in guanosine the base is not definitely fixed with respect to the ribose. Calculation on different anomeric nucleosides of adenosine and uridine shows that the configuration at the anomeric carbon C-1' determines the sign of the optical rotation. The configuration at C-2' influences the glycosidic angular dependence of rotational strength more profoundly than that at C-3' and C-5'. These results are in good agreement with experiments. The signs and magnitude of the calculated rotational strengths are in good agreement with experiment for the anti conformation of all the isomers of adenosine. As the conformation of the nucleosides in B-form DNA is quite different from the anti form, we calculate that the rotational strengths of the nucleosides in the polynucleotide are very different from those in solution.

Many workers have measured the circular dichroism (CD) and optical rotatory dispersion (ORD) of polynucleotides, and have shown that optical activity is an important tool for conformational assignments. Theories have been developed to facilitate the interpretation of the spectra of polynucleotides.<sup>3,4</sup> However, in these theories, the CD and ORD of the monomer units themselves have been ignored. Recently, experimental and theoretical studies of the optical activity and conformation of nucleosides have appeared.<sup>5-7</sup> In

particular, an extended series of articles by Miles *et al.*,<sup>8</sup> have investigated this problem in detail.

In the present paper we have used an improved version of Kirkwood polarizability theory to include the presence of a classical polarizability near a quantum system. The rotational strengths of mononucleosides are calculated using transition monopoles on the bases interacting with polarizable bonds of the sugars. We try to examine the calculations critically. Three different sets of transition monopoles have been employed with various degrees of success. The effect of different values of bond polarizability and variation of the positions of furanosyl OH groups have also been examined. The calculated rotational strengths as a function of the glycosidic angle are in qualitative agreement

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# Synthesis of Guanosine 5'-Di- and -Triphosphate Derivatives with Modified Terminal Phosphates: Effect on Ribosome-Elongation Factor G-Dependent Reactions<sup>†</sup>

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**ABSTRACT:** A series of GTP and GDP analogues modified in the terminal phosphate has been synthesized and their activities were investigated in elongation factor G dependent reactions. All of the analogues, with the exception of guanosine 5'-O-(3-thiotriphosphate), were not hydrolyzed by EF-G and ribosomes, but were competitive inhibitors of the ribosome-dependent EF-G GTPase. The most active inhibitors were *P*<sup>3</sup>-fluoro *P*<sup>1</sup>-5'-guanosine triphosphate and *P*<sup>3</sup>-methyl *P*<sup>1</sup>-5'-guanosine triphosphate with a *K*<sub>i</sub> of  $1.0 \times 10^{-6}$  and  $2.5 \times 10^{-6}$  M, respectively. The activity of the GTP alkyl ester derivatives decreased with increasing number of carbon atoms in the side chain. GTP analogues were much more effective inhibitors than the corresponding GDP derivatives. This points out the necessity of the presence of at least three negative charges in the phosphate chain of the nucleotide for an effective interaction with the active site of

the ribosomal EF-G GTPase. Guanosine 5'-O-(3-thiotriphosphate), which was hydrolyzed at one-third the rate of GTP, was able to support poly(U)-directed poly(phenylalanine) polymerization. Possible mechanisms of ribosome-EF-G GTP hydrolysis that arise from our results are discussed. Activity of the nucleotide analogues in EF-G-ribosome complex formation compared well with their ability to inhibit ribosome-dependent EF-G GTPase, *P*<sup>3</sup>-fluoro *P*<sup>1</sup>-5'-guanosine triphosphate and *P*<sup>3</sup>-methyl *P*<sup>1</sup>-5'-guanosine triphosphate being again the most effective ones. The stabilizing action of fusidic acid on the EF-G-ribosome complex formation induced by the various nucleotides could not be correlated to any of the structural modifications of the substrate. Guanylyl methylene diphosphonate was displaced more readily than GDP from the EF-G-ribosome complex by GTP analogues insensitive to fusidic acid.

The translation of the genetic message on the ribosome is catalyzed by various enzymatic reactions which utilize GTP as energy donor (for review, see Lucas-Lenard and Lipmann, 1971; Haselkorn and Rothman-Denes, 1973). A systematic investigation with GTP analogues seemed to be appropriate for a better understanding of the mechanism of GTP hydrolysis in these reactions. We approached this problem by trying to synthesize nonhydrolyzable GTP analogues other than GMPP(CH<sub>2</sub>)P and GMPP(NH)P (Herstey and Monro, 1966; Eckstein et al., 1971). Our finding that *P*<sup>3</sup>-methyl *P*<sup>1</sup>-5'-guanosine triphosphate was not hydrolyzed by the ribosome-EF-G GTPase prompted us to synthesize a series of GTP  $\gamma$ - and GDP  $\beta$ -phosphate derivatives (Figure 1) and to study their properties in EF-G-dependent reactions. EF-G, an essential component of the

elongation process, catalyzes a ribosome-dependent GTP hydrolysis which is involved in the advancement of mRNA and the simultaneous translocation of the polypeptide chain from the acceptor to the peptidyl site of the ribosome (Nishizuka and Lipmann, 1966; Haenni and Lucas-Lenard, 1968; Erbe et al., 1969). Ribosome-EF-G-dependent reactions have been extensively studied and represent an excellent tool to compare the effects of structural modifications of GTP and GDP; at the same time this approach allows the investigation of functional aspects of EF-G activities that are not yet fully understood.

## Experimental Procedure

**Materials.** Alkaline phosphatase (calf intestine, 1 mg/ml, 350 U/mg), snake venom phosphodiesterase (1 mg/ml, 1.5 U/mg), GMPP(CH<sub>2</sub>)P, GMPP(NH)P, GDP( $\beta$ NH<sub>2</sub>), GDP, GTP, ADP, ATP, and poly(U) were purchased from Boehringer. [<sup>3</sup>H]GMPP(CH<sub>2</sub>)P (6600 Ci/mol), [<sup>3</sup>H]GDP (5000 Ci/mol), and [<sup>3</sup>H]phenylalanine (20000 Ci/mol) were supplied by Amersham. [<sup>35</sup>S]GTP( $\gamma$ S) was synthesized by the enzymatic phosphate exchange reaction described for ATP (Glynn and Chappell, 1964) using GTP( $\gamma$ S) (Goody and Eckstein, 1971) and [<sup>35</sup>S]thiophosphate (F. Eckstein, unpublished). [<sup>32</sup>P]GTP was prepared as described by Sander et al. (1972). Purity of commercial nucleotides was monitored by chromatography on PEI-cellulose. GMPP(NH)P was highly contaminated with GDP( $\beta$ NH<sub>2</sub>) and was purified by electrophoresis (see Other Methods). Dithiothreitol was from Calbiochem, Aquasol from New England Nuclear, and 2,5-diphenyloxazole from Packard. Fusidic acid was a gift of Leo Pharmaceutical Products, Denmark. Sucrose (Merck, für biochem-

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<sup>‡</sup> Abbreviations used are: EF-G, elongation factor G; EF-T, the complex formed by elongation factors Tu and Ts; GMPP(CH<sub>2</sub>)P, guanylyl methylene diphosphonate; GMPP(NH)P, guanylyl iminodiphosphate; GDP<sup>ox-red</sup>, guanosine 5'-diphosphate with the C(2')-C(3') bond oxidized by periodate and reduced by borohydride; Cbz, benzylloxycarbonyl; poly(U), poly(uridylic acid). The modified GTP's, GDP's, and ATP's are abbreviated as, e.g., GTP( $\gamma$ X), where  $\gamma$  (or  $\beta$ ) refers to the terminal phosphate residue (see Figure 1), and X is the substituent, expressed in standard symbols, except for 4-azophenyl. With the exception of F, S, and NH<sub>2</sub>, the substituents are attached to P through an oxygen.

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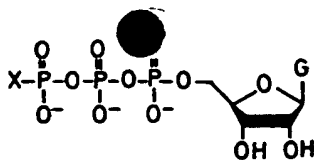


FIGURE 1: Representative structural formula for some GTP analogues. G = guanine; for GTP, X = OH; GTP( $\gamma$ F), X = F; GTP( $\gamma$ S), X = SH; GTP( $\gamma$ Me), X = OCH<sub>3</sub>; GTP( $\gamma$ Ph), X = OC<sub>6</sub>H<sub>5</sub>; GTP( $\gamma$ EtNHAc), X = OC<sub>2</sub>H<sub>4</sub>NHCOCH<sub>3</sub>.

ische und mikrobiologische Zwecke) was treated with diethyl pyrocarbonate to inactivate RNase (Solymosy et al., 1968). All other chemicals were reagent grade.

Elongation factors and ribosomes were isolated from *Escherichia coli* BT2<sup>r</sup> or A19. EF-G and EF-T were purified to homogeneity by modification of the published procedures (Parmeggiani, 1968; Parmeggiani et al., 1971): filtration on Sephadex G-200 was replaced by chromatography on DEAE-Sephadex A-50. The factors were stored at -25° in 50% glycerol, 50 mM Tris-HCl (pH 7.8 at 20°), and 2 mM dithiothreitol; 1  $\mu$ g of EF-G and EF-T was taken to correspond to 12 and 15 pmol, respectively (Sander et al., 1975). For preparation of [<sup>3</sup>H]EF-G, bacteria were grown in a medium containing in 4 l.: 20 g of NaCl, 42.8 g of KH<sub>2</sub>PO<sub>4</sub>, 8 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.8 g of MgSO<sub>4</sub>, 2 mg of FeSO<sub>4</sub>, 2 g of glutamic acid, and 40 g of glucose (adjusted to pH 7 with NH<sub>4</sub>OH). 10 mCi of [<sup>3</sup>H]glycine or [<sup>3</sup>H]valine (Amersham, 2800 Ci/mol) was added during the early-log-phase to the medium. The cells were harvested as soon as the residual radioactivity of the medium, after centrifugation, indicated that most of the amino acid had entered the cells. [<sup>3</sup>H]EF-G was extracted and purified as above. Ribosomes were prepared by suspending 0.5–1.0 g of crude ribosomes (Parmeggiani et al., 1971) in 23 ml of 0.5 M NH<sub>4</sub>Cl, 20 mM Tris-HCl (pH 7.8), and 10 mM MgCl<sub>2</sub>, and sedimented for 6 hr at 225000g in a Spinco 60 Ti rotor through 12 ml of 18% sucrose in the same buffer. This procedure was repeated two to three times; 1 A<sub>260</sub> unit of ribosomes was assumed to equal 25 pmol (Hill et al., 1970). Protein concentrations were determined according to Lowry et al. (1951) using serum bovine albumin as a standard. Partially purified tRNA<sup>Phe</sup> from *E. coli*, accepting 152 pmol of phenylalanine/A<sub>260</sub> unit of tRNA, was obtained from tRNA (Schwarz) by chromatography on benzoyleated DEAE-cellulose (Gillam et al., 1967) and charged with [<sup>3</sup>H]phenylalanine as described by Chinali and Parmeggiani (1973).

#### Synthesis of Analogues

**P<sup>3</sup>-Methyl P<sup>1</sup>-5'-Guanosine Triphosphate.** GTP (disodium salt, 0.57 g, 1 mmol) was converted into its pyridinium salt by passage over a Merck I ion exchange column (pyridinium form). For the conversion into the tri-*n*-octylammonium salt the pyridinium salt was dissolved in methanol containing tri-*n*-octylamine (0.84 ml, 2 mmol) by gentle heating with a fan, evaporated, and dried by repeated (three times) evaporation with pyridine. It was dissolved in dry dioxane (2 ml) and allowed to react for 2 hr at room temperature with diphenyl phosphorochloridate (0.3 ml) and tri-*n*-butylamine (0.45 ml). The solvents were then removed by evaporation, ether (30 ml) and petroleum ether (80 ml) were added, and the mixture was left at 0° for 30 min. The supernatant was decanted; the residue was dissolved in dioxane (3 ml) and evaporated. The residue was dissolved in pyridine (2 ml) and methanol (1 ml). After reaction for 2 hr at room temperature with stirring, the sol-

vents were removed by evaporation. The residue was extracted with water and chromatographed on a DEAE-cellulose column (Whatman DE-52; 2.5 × 50 cm) with a linear gradient of water–0.3 M triethylammonium bicarbonate (1.5 l. each). The product was eluted at about 0.25 M buffer. The combined fractions were evaporated and the buffer was removed by repeated evaporation of the residue with methanol. Yield, 4000 A<sub>252</sub> units, 0.29 mmol; <sup>1</sup>H NMR (D<sub>2</sub>O)  $\delta$  4.36 ppm (*J*<sub>P-O-C-H</sub> = 12 Hz, d, CH<sub>3</sub>; on <sup>31</sup>P-decoupling, s); <sup>31</sup>P NMR (D<sub>2</sub>O, pH ~10, H-decoupled)  $\delta$  9.10 ppm (d,  $\gamma$ -P), 10.73 (d,  $\alpha$ -P), 21.60 (t,  $\beta$ -P). Electrophoretic mobility at pH 3.5: *R*<sub>GMP</sub> 0.47; *R*<sub>GDP</sub> 0.82; *R*<sub>GTP</sub> 1.0; *R*<sub>GTP( $\gamma$ Me)</sub> 1.0. Anal. Calcd for C<sub>29</sub>H<sub>63</sub>N<sub>8</sub>O<sub>14</sub>P<sub>3</sub>: N, 13.33; P, 11.05. Found: N, 14.10; P, 11.25.

The  $\gamma$ -phosphate ethyl, propyl, and butyl esters of GTP were prepared analogously as well as the  $\gamma$ -phosphate methyl ester of ATP. Electrophoretic mobility at pH 3.5: *R*<sub>AMP</sub> 0.35; *R*<sub>ADP</sub> 0.77; *R*<sub>ATP</sub> 1.0, *R*<sub>ATP( $\gamma$ Me)</sub> 1.0. <sup>1</sup>H NMR (D<sub>2</sub>O)  $\delta$  3.58 ppm (*J*<sub>P-O-C-H</sub> = 12 Hz, d, CH<sub>3</sub>; on <sup>31</sup>P-decoupling, s). Anal. Calcd for C<sub>29</sub>H<sub>63</sub>N<sub>8</sub>O<sub>13</sub>P<sub>3</sub>·3H<sub>2</sub>O: N, 12.75; P, 10.57. Found: N, 12.52; P, 9.91. The  $\beta$ -phosphate methyl ester of GDP was synthesized in the same way using GDP instead of GTP [electrophoretic mobility at pH 3.5: *R*<sub>GDP</sub> 1.05; *R*<sub>GTP</sub> 1.15; *R*<sub>GDP( $\beta$ Me)</sub> 1.0; <sup>1</sup>H NMR (D<sub>2</sub>O)  $\delta$  4.36 ppm (*J*<sub>P-O-C-H</sub> = 12 Hz, d, CH<sub>3</sub>; on <sup>31</sup>P-decoupling, s). Anal. Calcd for C<sub>23</sub>H<sub>47</sub>N<sub>7</sub>O<sub>4</sub>P<sub>2</sub>: N, 14.19; P, 9.39. Found: N, 13.02; P, 8.80].

**P<sup>3</sup>-Aminoethyl P<sup>1</sup>-5'-Guanosine Triphosphate.** *N*-Cbz-aminoethyl Phosphate. *N*-Cbz-ethanolamine (Rose, 1947) (2 mmol, 400 mg) was dissolved in triethyl phosphate (5 ml) and POCl<sub>3</sub> (0.3 ml) added at 0°. After standing for 2.5 hr at 4°, aqueous barium acetate solution (10%, 20 ml) was added and after 5 min the material precipitated with triethylamine (2 ml). After addition of ethanol (60 ml) it was centrifuged; the residue was extracted with 70% aqueous ethanol (three times with 40 ml) and finally with water (5 × 50 ml). The aqueous solutions were combined and concentrated under reduced pressure to about 50 ml. The Ba<sup>2+</sup> salt precipitated and was collected by filtration. Yield, 180 mg (23%). Anal. Calcd for C<sub>10</sub>H<sub>12</sub>NO<sub>5</sub>PBa: N, 3.54; P, 7.85. Found: N, 3.15; P, 7.96.

**P<sup>3</sup>-(*N*-Cbz-aminoethyl) P<sup>1</sup>-5'-Guanosine Triphosphate.** The Ba<sup>2+</sup> salt of *N*-Cbz-aminoethyl phosphate (250 mg, 0.63 mmol) was converted into the pyridinium salt by passage over a Merck I ion exchanger (pyridinium form). After evaporation to dryness this salt was converted to the tri-*n*-octylammonium salt by addition of tri-*n*-octylamine (0.26 ml) and methanol (ca. 50 ml), and gentle heating with a fan. After a clear solution had been obtained it was evaporated to dryness and dried by two evaporations with dry pyridine and one with dry HCONMe<sub>2</sub>. The residue was taken up in dioxane (2 ml) and diphenyl phosphorochloridate (0.2 ml) and tri-*n*-butylamine (0.36 ml) were added. After 2 hr at room temperature, ether (20 ml) and petroleum ether (bp 40–60°, 40 ml) were added. After 30 min at 0° the supernatant was decanted, the residue dissolved in dioxane (5 ml) and evaporated, and the residue dissolved in HCONMe<sub>2</sub> (5 ml). This solution was added to the tri-*n*-octylammonium salt of GDP (1 mmol) which had been dried by repeated evaporations with pyridine and HCONMe<sub>2</sub>. After 2 hr at room temperature the solution was evaporated; the residue was extracted with water and chromatographed on a DEAE-Sephadex A-25 column (2 × 30 cm) with a linear gradient of 1.5 l. each of water and 0.5 M triethylammonium bicarbonate (pH 7.5). The product



was eluted at about 0.3 *M* buffer. The fractions were pooled and evaporated to dryness and the buffer was removed by repeated evaporation with methanol. Yield, 4000 *A*<sub>252</sub> units (31%). Electrophoretic mobility at pH 3.5: *R*<sub>GMP</sub> 0.66; *R*<sub>GDP</sub> 1.22; *R*<sub>GTP(γR)</sub> 1.0.

***P*<sup>3</sup>-Aminoethyl *P*<sup>1</sup>-5'-Guanosine Triphosphate.** *P*<sup>3</sup>-(*N*-Cbz-aminoethyl) *P*<sup>1</sup>-5'-guanosine triphosphate (2000 *A*<sub>252</sub> units, 0.15 mmol) was dissolved in 5% aqueous acetic acid (5 ml) and 10% palladium on charcoal (60 mg) added. The vessel was filled with hydrogen to a slight excess above atmospheric pressure (50 cm of H<sub>2</sub>O) and hydrogenation carried out for 2 hr. The catalyst was filtered off; the filtrate was evaporated to dryness and chromatographed on a DEAE-Sephadex A-25 column (1.5 × 20 cm) with a linear gradient of 500 ml each of H<sub>2</sub>O and 0.5 *M* triethylammonium bicarbonate (pH 7.5). The product was eluted at about 0.25 *M* buffer. Yield, 1800 *A*<sub>252</sub> units. Electrophoretic mobility at pH 3.5: *R*<sub>GMP</sub> 0.77; *R*<sub>GDP</sub> 1.39; *R*<sub>GTP(γEINH<sub>2</sub>)</sub> 1.0.

***P*<sup>3</sup>-*N*-Acetylaminoethyl *P*<sup>1</sup>-5'-Guanosine Triphosphate.** To a solution of the tri-*n*-octylammonium salt of *P*<sup>3</sup>-aminoethyl *P*<sup>1</sup>-5'-guanosine triphosphate (450 *A*<sub>252</sub> units) in methanol (2 ml) a solution of acetyl-*N*-hydroxysuccinimide (157 mg, 1 mmol) (De Groot et al., 1966) in acetone (2 ml) was added. After reaction for 1 hr at room temperature the solution was evaporated; the residue was extracted with water and chromatographed on a DEAE-Sephadex A-25 column (1.5 × 20 cm) with a linear gradient of 500 ml each of water and 0.5 *M* triethylammonium bicarbonate (pH 7.5). The material was eluted at about 0.35 *M* buffer. Yield, 350 *A*<sub>252</sub> units. Electrophoretic mobility at pH 3.5: *R*<sub>GDP</sub> 0.95; *R*<sub>GTP(γEINHAc)</sub> 1.0; <sup>1</sup>H NMR (D<sub>2</sub>O) δ 2.0 ppm (s, CH<sub>3</sub>). Anal. Calcd for C<sub>32</sub>H<sub>68</sub>N<sub>9</sub>O<sub>15</sub>P<sub>3</sub>: N, 13.82; P, 10.19. Found: N, 14.26; P, 10.78.

The corresponding ester of ATP was synthesized in the same way.

***P*<sup>2</sup>-Phenyl *P*<sup>1</sup>-5'-Guanosine Diphosphate.** This compound was synthesized as described for *P*<sup>3</sup>-(*N*-Cbz-aminoethyl) *P*<sup>1</sup>-5'-guanosine triphosphate using phenyl phosphate (2 mmol) and GMP (1 mmol) as starting materials. Yield, 3900 *A*<sub>252</sub> units. Electrophoretic mobility at pH 3.5: *R*<sub>GMP</sub> 0.59; *R*<sub>GDP</sub> 1.15; *R*<sub>GDP(βPh)</sub> 1.0; λ<sub>max</sub><sup>H<sub>2</sub>O</sup> 252 nm (ε 13300); <sup>1</sup>H NMR (D<sub>2</sub>O) δ 7.65 ppm (s, 5 H); <sup>31</sup>P NMR (D<sub>2</sub>O) δ 11.57 ppm (d) and 16.16 (d). Anal. Calcd for C<sub>28</sub>H<sub>47</sub>N<sub>7</sub>O<sub>11</sub>P<sub>2</sub>: N, 13.62; P, 8.60. Found: N, 13.65; P, 8.47.

***P*<sup>3</sup>-Phenyl *P*<sup>1</sup>-5'-Guanosine Triphosphate.** This compound was synthesized by activation of phenyl phosphate (2 mmol) with carbonyldiimidazole, in analogy to the work of Barker et al. (1972), and reaction with GDP (1 mmol). Purification was carried out by chromatography on DEAE-Sephadex as described for GTP(γMe). Yield, 300 *A*<sub>252</sub> units. Electrophoretic mobility at pH 3.5: *R*<sub>GTP</sub> 1.0; *R*<sub>GTP(γPh)</sub> 1.0; *R*<sub>GDP</sub> 0.85; *R*<sub>GDP(βPh)</sub> 0.85. <sup>1</sup>H NMR (D<sub>2</sub>O) δ 7.65 ppm (s, 5 H); <sup>31</sup>P NMR (D<sub>2</sub>O) δ 11.6 ppm (d); 16.2 (d); 23.3 (t). Anal. Calcd for C<sub>34</sub>H<sub>63</sub>N<sub>9</sub>O<sub>14</sub>P<sub>3</sub>: N, 12.43; P, 10.31. Found: N, 11.98; P, 10.68. In this synthesis 330 *A*<sub>252</sub> units of GDP(βPh) was produced as well.

***P*<sup>3</sup>-Fluoro *P*<sup>1</sup>-5'-Guanosine Triphosphate and *P*<sup>2</sup>-Fluoro *P*<sup>1</sup>-5'-Guanosine Diphosphate.** These compounds were prepared following the procedure given for the synthesis of *P*<sup>3</sup>-(*N*-Cbz-aminoethyl) *P*<sup>1</sup>-5'-guanosine triphosphate with 1 mmol of GDP and using phosphorofluoridic acid instead of *N*-Cbz-aminoethyl phosphate. The reaction with GDP led to the di- as well as the triphosphate derivative. This method was also used for the synthesis of ATP(γF) by

Haley and Yount (1972). Yield, 1320 *A*<sub>252</sub> units of the diphosphate derivative and 1350 *A*<sub>252</sub> units of the triphosphate derivative.

Diphosphate derivative: <sup>31</sup>P NMR (D<sub>2</sub>O) δ 11.05 ppm (d, *J* = 19.7 Hz); 17.94 ppm (d, *J*<sub>P-F</sub> = 450 Hz; d, *J*<sub>P-P</sub> = 19.7 Hz). Anal. Calcd for C<sub>22</sub>H<sub>42</sub>N<sub>7</sub>O<sub>10</sub>P<sub>2</sub>F: N, 15.56; P, 9.85; F, 2.70. Found: N, 15.14; P, 9.56; F, 2.93.

Triphosphate derivative: <sup>31</sup>P NMR (D<sub>2</sub>O) δ 11.87 (d, *J* = 19.0 Hz) and 18.33 ppm (d, *J*<sub>P-F</sub> = 450 Hz; d, *J*<sub>P-P</sub> = 19.0 Hz). Anal. Calcd for C<sub>28</sub>H<sub>60</sub>N<sub>8</sub>O<sub>13</sub>P<sub>3</sub>F: N, 13.52; P, 11.21; F, 2.29. Found: N, 14.06; P, 11.20; F, 2.2.

[<sup>3</sup>H]GTP(γF) was synthesized in the same way starting with [<sup>3</sup>H]GDP.

All of these analogues were stable against alkaline phosphatase and were degraded to GMP by snake venom phosphodiesterase. To eliminate contaminations of GTP or GDP, all modified nucleotides were treated with alkaline phosphatase. Approximately 1 μmol of substrate was incubated in 50 μl of 0.1 *M* Tris-HCl (pH 8.5) containing 5 μl of alkaline phosphatase for 2 hr at 37°. Recovery of the nucleotide was achieved by electrophoresis or column chromatography on DEAE-Sephadex A-25. All of these nucleotides were stable for several months when stored at -35° as frozen aqueous solutions even when repeatedly thawed and frozen.

**Other Methods.** Nuclear magnetic resonance spectra were recorded on a Bruker Physik HFX 60 spectrometer equipped with a Nicolet FT 1074 averaging system with Me<sub>4</sub>Si as internal standard for <sup>1</sup>H spectra and dilute H<sub>3</sub>PO<sub>4</sub> as external standard for <sup>31</sup>P spectra. Chemical shifts are given in δ units (ppm). Electrophoresis was carried out on paper, Schleicher & Schüll, 2043b (washed), at pH 3.5 (0.05 *M* ammonium formate) for 90 min and 30 V/cm. Chromatography on PEI-cellulose (Polygram CEL 300 PEI/UV, Macherey & Nagel) was done in 0.75 *M* KH<sub>2</sub>PO<sub>4</sub> at pH 3.5 for 2 hr. For determination of labeled compounds the PEI-cellulose plates were cut in pieces (1.5 × 1.5 cm) and measured in a toluene scintillation fluid. Radioactivity was measured with a Packard 3380 scintillation spectrometer using either 5 ml of a toluene scintillation fluid containing 5 g of 2,5-diphenyloxazole/l. or 10 ml of Aquasol.

**Assay of EF-G-Dependent Ribosomal Activities.** Ribosome-dependent EF-G GTPase activity was tested by measuring the liberated inorganic phosphate (Parmeggiani et al., 1971). Stable EF-G-nucleotide-ribosome complexes were isolated by chromatography on a Sephadex G-200 column (25 × 0.7 cm) equilibrated with 50 mM Tris-HCl (pH 7.8), 20 mM MgCl<sub>2</sub>, 80 mM NH<sub>4</sub>Cl, and 1 mM dithiothreitol, or by sedimentation through 3 ml of a 10% sucrose cushion containing 50 mM Tris-HCl (pH 7.8), 20 mM MgCl<sub>2</sub>, 80 mM NH<sub>4</sub>Cl, and 1 mM dithiothreitol in a Spinco 50 Ti rotor. The centrifuge tubes were filled up with paraffin oil; centrifugation was done at 134000g for 150 min. The supernatant was rapidly removed and the small transparent ribosomal pellet rinsed with 0.1 ml of a solution containing 20 mM Tris-HCl (pH 7.8), 20 mM MgCl<sub>2</sub>, 30 mM KCl, 30 mM NH<sub>4</sub>Cl, and 1 mM dithiothreitol, and then suspended in 0.1 ml of the same solution by gentle shaking for 30-60 min. The amount of EF-G bound to ribosomes was calculated from the GTPase activity of the isolated ribosomal complex, corrected for the activity of ribosomes isolated under the same conditions in the absence of nucleotides. To test in a range linear to the amount of factor bound, the molar ratio of ribosomes to EF-G was at least 30

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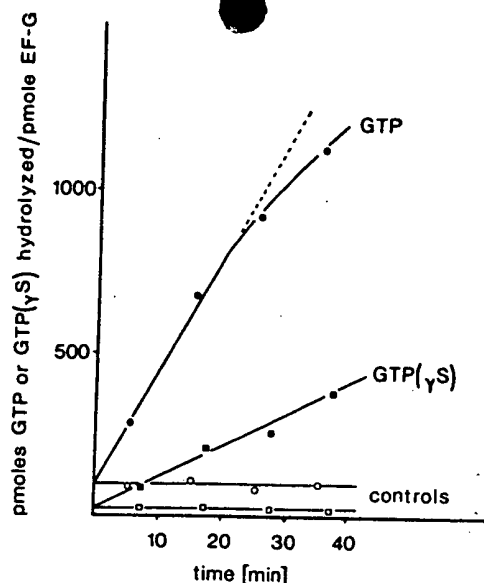


FIGURE 2: Rate of ribosome-EF-G-dependent hydrolysis of GTP and GTP( $\gamma$ S). The reaction mixture contained in 0.020 ml: 20 mM Tris-HCl (pH 7.8), 15 mM MgCl<sub>2</sub>, 80 mM NH<sub>4</sub>Cl, 1 mM dithiothreitol, 19 pmol of ribosomes, 29 pmol of EF-G when present, and 1.7 mM [<sup>32</sup>P]GTP (1.5 Ci/mol) or 4.4 mM [<sup>35</sup>S]GTP( $\gamma$ S) (1 Ci/mol). The reaction mixture was incubated at 30°. Aliquots of 4  $\mu$ l were taken at the indicated time intervals and analyzed on PEI-cellulose (see Experimental Procedure). [<sup>32</sup>P]GTP plus (●) and minus (○) EF-G; [<sup>35</sup>S]GTP( $\gamma$ S) plus (■) and minus (□) EF-G.

(Chinali and Parmeggiani, 1973). This was obtained by adding untreated ribosomes to the assay system.

Chromatography of [<sup>3</sup>H]EF-G-ribosome complexes was done on Sepharose 6B columns (0.5  $\times$  7 cm) equilibrated with 20 mM Tris-HCl (pH 7.8), 10 mM MgCl<sub>2</sub>, 10 mM NH<sub>4</sub>Cl, and, when present, 0.2 mM fusidic acid. For the calculation of the amount of nucleotide bound in the complex, radioactivity of 0.10-ml aliquots of the 0.15-ml fractions, containing about 15 pmol of ribosomes, was measured in 10 ml of Aquasol and the amount of bound EF-G calculated on the basis of a 1:1 EF-G/nucleotide ratio. The ability of the various nucleotide analogues to displace GMPP(CH<sub>2</sub>)P or GDP bound to EF-G-ribosome complexes was assayed by determining the amount of [<sup>3</sup>H]GMPP(CH<sub>2</sub>)P or [<sup>3</sup>H]GDP retained on nitrocellulose filters (Sartorius, 0.45- $\mu$ m pore size) in the presence of different concentrations of the nucleotide analogues. For details of experimental conditions see legends.

## Results

**Properties of the  $\gamma$ -Phosphate Analogues of GTP.** The  $\gamma$ -phosphate methyl ester of ATP has previously been prepared by Wehrli et al. (1964) by condensation of ATP and methanol with dicyclohexylcarbodiimide. For the synthesis of the  $\gamma$ -phosphate alkyl esters of GTP and ATP we preferred activation of the nucleotide with diphenyl phosphorochloridate (Michelson, 1964) and subsequent reaction with the corresponding alcohol. This method has also been found to furnish good yields in the synthesis of other nucleoside triphosphate derivatives (Goody, Fröhlich, Walter and Schirmer; personal communication). The <sup>31</sup>P NMR spectrum of the methyl ester of GTP only showed a significant shift of the  $\gamma$  phosphorus from  $\delta$  5.08 ppm for GTP to  $\delta$  9.10 ppm. Together with the resistance of this compound to alkaline phosphatase this indicates that esterification occurred at the terminal phosphate. This method of synthesis

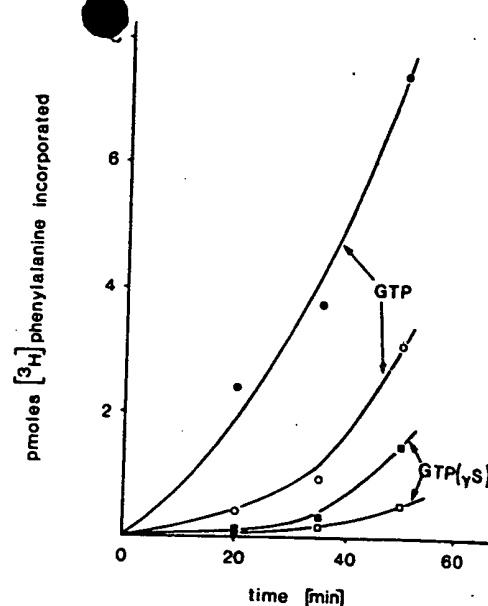


FIGURE 3: Rate of GTP( $\gamma$ S)- and GTP-catalyzed poly(phenylalanine) synthesis. The reaction mixture contained in a final volume of 0.10 ml: 50 mM Tris-HCl (pH 7.8), 9 mM MgCl<sub>2</sub>, 53 mM KCl, 1 mM dithiothreitol, 28 pmol of ribosomes, 6.2  $\mu$ g of poly(U), 200 pmol of [<sup>3</sup>H]Phe-tRNA<sup>Phe</sup> (8030 Ci/mol), 60 pmol of EF-T, and 7 pmol of EF-G in the presence of  $6.6 \times 10^{-5}$  M (○) and  $3.3 \times 10^{-4}$  M (●) GTP or  $6.6 \times 10^{-5}$  M (□) and  $3.3 \times 10^{-4}$  M (■) GTP( $\gamma$ S); 25- $\mu$ l samples were taken from the reaction mixture at the indicated time intervals. The activity was determined by measuring the incorporation of [<sup>3</sup>H]phenylalanine into hot trichloroacetic acid insoluble material using GFA filters (Häenni and Chapeville, 1966).

was only applicable to liquid alcohols. For the preparation of derivatives of the aminoethyl ester of nucleoside triphosphates an approach was chosen which had been successful in the synthesis of ATP( $\gamma$ S) (Goody and Eckstein, 1971) and the *p*-nitrophenyl ester of ATP (Berglund and Eckstein, 1972). *N*-Cbz-aminoethyl phosphate was activated with diphenyl phosphorochloridate and allowed to react with a nucleoside diphosphate. Removal of the NH<sub>2</sub>-protecting group yields a compound which can be selectively acylated using the esters of *N*-hydroxysuccinimide (De Groot et al., 1966). For the synthesis of a similar compound, P<sup>2</sup>-(6-amino-1-hexyl) P<sup>1</sup>-5'-uridine diphosphate, Barker et al. (1972) gave preference to activation by carbonyldiimidazole while protecting the amino group as its trifluoroacetyl derivative.

P<sup>3</sup>-Fluoro P<sup>1</sup>-5'-adenosine triphosphate, ATP( $\gamma$ F), had been synthesized earlier by Haley and Yount (1972) as an ATP analogue resistant to alkaline phosphatase, myosin, and hexokinase. We found that in the synthesis of GTP( $\gamma$ F) substantial amounts of GDP( $\beta$ F) were formed. This might be due to hydrolysis of the nucleoside diphosphate to the nucleoside monophosphate prior to the condensation reaction. Attempts to synthesize this analogue by reaction of the nucleoside triphosphate with dinitrofluorobenzene in analogy to a similar reaction with thymidine 5'-monophosphate (Wittmann, 1963) were not successful.

**Ability of the  $\gamma$ -Phosphate Analogues of GTP to be Hydrolyzed by EF-G and Ribosome and to Support Poly(U)-Directed Poly(phenylalanine) Synthesis.** The  $\gamma$ -phosphate analogues with the exception of GTP( $\gamma$ S) were found to be resistant to the hydrolytic action of EF-G and ribosomes as is the case with GMPP(CH<sub>2</sub>)P and GMPP(NH)P. This was measured in a reaction mixture containing in a final volume of 0.020 ml: 20 mM Tris-HCl (pH 7.8), 13 mM

Table I: Inhibition of Ribosome-Dependent EF-G GTPase by Various Nucleotides.

Nucleotide	$K_i$ [M]	Nucleotide	$K_i$ [M]
GDP	$3.6 \times 10^{-5}$	GTP( $\gamma$ Bu)	$7.5 \times 10^{-5}$
GMPP(CH <sub>2</sub> )P	$3.9 \times 10^{-5}$	GTP( $\gamma$ EtNH <sub>2</sub> )	$4.4 \times 10^{-5}$
GMPP(NH)P	$4.3 \times 10^{-5}$	GTP( $\gamma$ EtNHAc)	$4.6 \times 10^{-5}$
GTP( $\gamma$ S)	$1.3 \times 10^{-5}$	GTP( $\gamma$ Ph)	$2.1 \times 10^{-5}$
GTP( $\gamma$ F)	$1.0 \times 10^{-5}$	GDP( $\beta$ NH <sub>2</sub> )	$2.4 \times 10^{-5}$
GTP( $\gamma$ Me)	$2.5 \times 10^{-5}$	GDP( $\beta$ F)	$4.0 \times 10^{-5}$
GTP( $\gamma$ Et)	$4.5 \times 10^{-5}$	GDP( $\beta$ Me)	$2.8 \times 10^{-5}$
GTP( $\gamma$ Pr)	$5.6 \times 10^{-5}$	GDP( $\beta$ Ph)	$1.0 \times 10^{-5}$

\*The reported  $K_i$  values were the average of several experiments. Under these conditions the  $K_m$  for GTP was  $5.6-6.6 \times 10^{-5}$  M. ATP( $\gamma$ Me) and ATP( $\gamma$ EtNHAc) showed no inhibition at concentrations up to  $1.0 \times 10^{-3}$  M. The reaction mixture contained in 0.075 ml: 50 mM Tris-HCl (pH 7.8), 13 mM MgCl<sub>2</sub>, 80 mM NH<sub>4</sub>Cl, 1 mM dithiothreitol, 55 pmol of ribosomes, 14 pmol of EF-G, 0.1  $\mu$ Cl of [<sup>32</sup>P]GTP, GTP in five different concentrations (0.02, 0.04, 0.08, 0.24, 1.20 mM), and nucleotide analogs in concentrations about twice their  $K_i$ . Incubation time: 15 min at 30°.

Table II: Nucleotide-Dependent Binding of [<sup>3</sup>H]EF-G to Ribosomes in the Presence and Absence of Fusidic Acid.<sup>a</sup>

	pmol of [ <sup>3</sup> H]EF-G/pmol of Ribosome	
	- Fusidic Acid	+ Fusidic Acid
GDP	0.15	0.66 <sup>b</sup>
GMPP(CH <sub>2</sub> )P	0.72	0.67 <sup>b</sup>
GMPP(NH)P	0.61	0.73
GTP( $\gamma$ S)	0.07	0.70 <sup>b</sup>
GTP( $\gamma$ F)	1.04	1.05 <sup>b</sup>
GTP( $\gamma$ Me)	0.81	0.77
GTP( $\gamma$ Et)	0.62	0.66
GTP( $\gamma$ Pr)	0.38	0.49
GTP( $\gamma$ Bu)	0.37	0.50
GTP( $\gamma$ EtNH <sub>2</sub> )	0.05	0.62
GTP( $\gamma$ EtNHAc)	0.04	0.31
GTP( $\gamma$ Ph)	0.00	0.32
GDP( $\beta$ F)	0.11	0.35
GDP( $\beta$ Me)	0.05	0.31
GDP( $\beta$ Ph)	0.08	0.11
GDP( $\beta$ NH <sub>2</sub> )	0.06	0.08
ATP( $\gamma$ Me)	0	0
ATP( $\gamma$ EtNHAc)	0	0

<sup>a</sup>Binding of [<sup>3</sup>H]EF-G to ribosomes was performed in a mixture containing in 0.020 ml: 20 mM Tris-HCl (pH 7.8), 10 mM MgCl<sub>2</sub>, 10 mM NH<sub>4</sub>Cl, 1 mM dithiothreitol, 75 pmol of ribosomes, 188 pmol of [<sup>3</sup>H]EF-G (66 Ci/mol), 1.5 mM fusidic acid when present, and 0.1 mM nucleotides. Incubation time: 10 min at 30°. For the isolation of the [<sup>3</sup>H]EF-G-ribosome complex on Sepharose 6B and determination of the radioactivity see Experimental Procedures.

<sup>b</sup>These results were confirmed by using <sup>3</sup>H-labeled nucleotides.

MgCl<sub>2</sub>, 80 mM NH<sub>4</sub>Cl, 1 mM dithiothreitol, 105 pmol of EF-G, 12 pmol of ribosomes, and 50 nmol of nucleotides. After incubation for 120 min at 30° no hydrolytic products were detected by thin-layer chromatography on PEI-cellulose, whereas GTP was already completely hydrolyzed after 15 min. As shown in Figure 2 the initial rate of GTP( $\gamma$ S) hydrolysis was found to be 28% of that of GTP. The activity of GTP( $\gamma$ S) was measured also in poly(U)-directed poly-(phenylalanine) synthesis: initial rate of GTP( $\gamma$ S)-catalyzed incorporation of [<sup>3</sup>H]phenylalanine was found to be 10-20% of that of the GTP-catalyzed reaction (Figure 3). This result does not differentiate between the EF-G- and the EF-Tu-GTPase activity. The effect of GTP( $\gamma$ S) on the latter activity is presently under investigation.

**Inhibition of the Ribosome-Dependent EF-G GTPase Activity.** All GTP analogues were found to be competitive inhibitors of the GTP hydrolysis catalyzed by EF-G and ribosomes. In Table I the  $K_i$  values for the different analogues, calculated from double reciprocal plots, are reported. GTP( $\gamma$ F) and GTP( $\gamma$ Me) are the most potent inhibitors even in comparison to GMPP(CH<sub>2</sub>)P and GMPP(NH)P. The inhibitory activity of the  $\gamma$ -phosphate esters of GTP decreased with increasing length of the side chain. GTP( $\gamma$ EtNH<sub>2</sub>) and GTP( $\gamma$ EtNHAc) showed a lower inhibition than the corresponding alcohol esters. Adenosine triphosphate analogues, tested to control the specificity of this reaction, did not show any inhibition. GDP, the product of the GTPase reaction, is an effective competitive inhibitor of the ribosome-dependent EF-G GTP hydrolysis (Conway and Lipmann, 1964) while the  $\beta$ -phosphate analogues of GDP, GDP( $\beta$ Me), GDP( $\beta$ F), GDP( $\beta$ Ph), and GDP( $\beta$ NH<sub>2</sub>) were found to be much less effective than GDP.

**Activity of the Nucleotide Analogues in the Formation of the EF-G-Ribosome Complex.** The nucleotide-dependent binding of EF-G to ribosomes was directly measured by using labeled elongation factor in the presence or absence of fusidic acid (Table II). GTP( $\gamma$ F) was the most effective nucleotide analogue inducing formation of a complex containing equimolar amounts of EF-G and ribosomes. In general the effect of most triphosphate analogues in the absence of fusidic acid followed a pattern similar to that observed in the inhibition of EF-G GTPase activity. In the presence of fusidic acid, GTP( $\gamma$ S) stimulated the binding of EF-G to

the same extent as that induced by GDP. Parallel experiments using either <sup>3</sup>H- or <sup>35</sup>S-labeled GTP( $\gamma$ S) showed that 80-90% of the nucleotide bound to the complex was in this case represented by GDP. Of the other analogues the greatest effect of fusidic acid was observed with GDP( $\beta$ F), GDP( $\beta$ Me), GTP( $\gamma$ Ph), and with the aminoalkyl ester derivatives of GTP. For comparison we studied also the effect of GMPP(CH<sub>2</sub>)P and GMPP(NH)P in this system. The GMPP(CH<sub>2</sub>)P-mediated binding of [<sup>3</sup>H]EF-G to ribosomes was slightly inhibited by fusidic acid in line with observations of other authors while that of GMPP(NH)P showed a stimulation (Eckstein et al., 1971). The amount of [<sup>3</sup>H]EF-G bound to ribosomes in the presence of GMPP(CH<sub>2</sub>)P was the same as the amount of labeled GMPP(CH<sub>2</sub>)P bound in the EF-G-ribosome complex (see Figure 4).

Nucleotide-dependent binding of EF-G to ribosomes was also investigated by isolation of stable EF-G-ribosome complexes on Sephadex G-200 and upon ultracentrifugation. The pattern of activity confirms the results obtained with [<sup>3</sup>H]EF-G. A 50-65% yield of complex was obtained with ultracentrifugation when compared to filtration on Sephadex G-200 (Table III). This was likely due to the hydrostatic pressure, which dissociates the less stable part of EF-G-ribosome complexes.

The possibility that [<sup>3</sup>H]GTP( $\gamma$ F) had been bound covalently to the EF-G-ribosome complex was excluded by the observation that addition of sodium dodecyl sulfate to a final concentration of 1% caused the release of the radioactivity otherwise retained on nitrocellulose filter (results not shown).

**Ability of the Nucleotide Analogues to Displace GDP or GMPP(CH<sub>2</sub>)P from EF-G-Ribosome Complexes.** To study in more detail the affinity and specificity of the GTP and GDP analogues for EF-G and ribosomes we investigated the ability of a few representative nucleotide analogues to

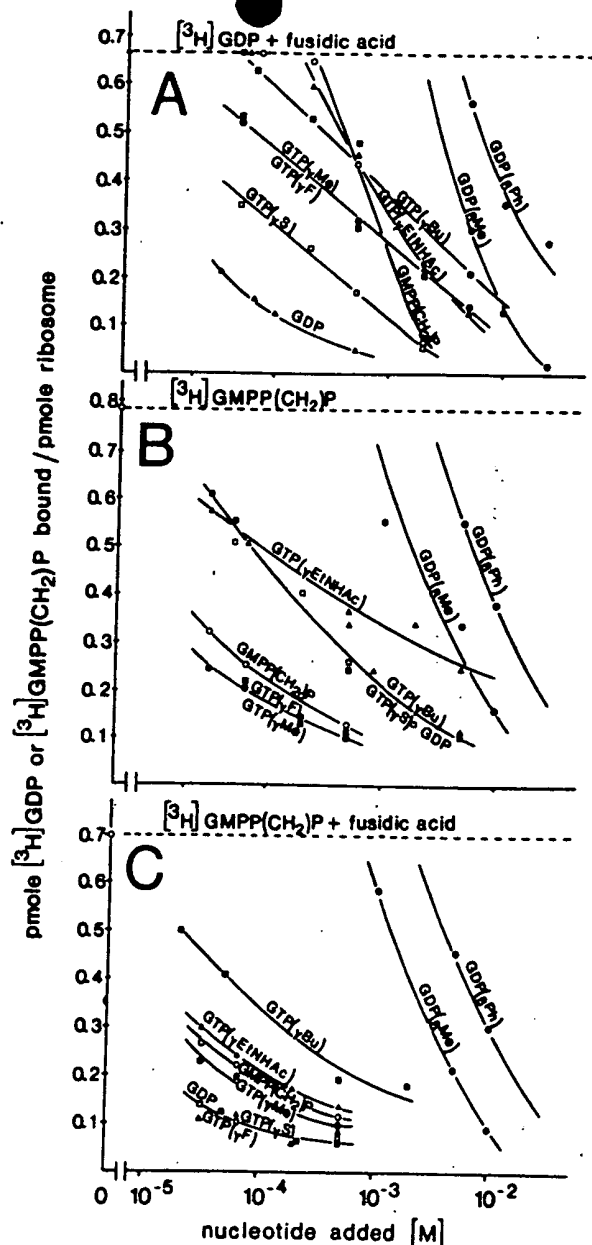


FIGURE 4: Ability of nucleotide analogues to displace GDP or GMPP(CH<sub>2</sub>)P from their respective EF-G-ribosome complexes. Complex formation was carried out in a reaction mixture of 0.030 ml containing in a final volume of 0.035 ml: 20 mM Tris-HCl (pH 7.8), 10 mM MgCl<sub>2</sub>, 10 mM NH<sub>4</sub>Cl, 1 mM dithiothreitol, 35 pmol of ribosomes, 125 pmol of EF-G, 1 mM fusidic acid when indicated, and  $2 \times 10^{-5}$  M [<sup>3</sup>H]GMPP(CH<sub>2</sub>)P or [<sup>3</sup>H]GDP (70 Ci/mol). After an incubation of 10 min at 30°, 5  $\mu$ l of unlabeled nucleotides was added to the final concentration indicated. After a second incubation of 15 min at 30°, an aliquot of 25  $\mu$ l was taken from the reaction mixture and pipetted under 10 ml of cold buffer (20 mM Tris-HCl, pH 7.8, 10 mM MgCl<sub>2</sub>, and 10 mM NH<sub>4</sub>Cl) onto the surface of a nitrocellulose filter. Suction was immediately applied so that filtration process lasted a few seconds. Filters were dried and radioactivity was measured in toluene scintillation fluid. The dashed lines indicate the level of [<sup>3</sup>H]GDP or [<sup>3</sup>H]GMPP(CH<sub>2</sub>)P bound to the EF-G-ribosome complex without addition of unlabeled nucleotide. The amount of [<sup>3</sup>H]GDP (0.15 pmol/pmol of ribosome) or [<sup>3</sup>H]GMPP(CH<sub>2</sub>)P (0.08 pmol/pmol of ribosome) bound in the absence of EF-G was subtracted. (Δ) GDP; (○) GMPP(CH<sub>2</sub>)P; (□) GTP(γS); (◻) GTP(γF); (●) GTP(γMe); (■) GTP(γBu); (Δ) GTP(γEtNHAc); (◊) GDP(βMe); (◊) GDP(βPh).

displace [<sup>3</sup>H]GDP and [<sup>3</sup>H]GMPP(CH<sub>2</sub>)P from their respective EF-G-ribosome complexes. To cover a wider range of conditions we have chosen both the fusidic acid-GDP

Table III: Nucleotide-Dependent Binding of EF-G to Ribosomes Measured as Ribosome-EF-G GTPase Activity.

	pmol of GTP Hydrolyzed/pmol of Ribosome in 10 min at 30°	Ultracentrifugation
Sephadex G-200		
—nucleotide	26.1	13.3
GDP	2.5	1.1
GMPP(CH <sub>2</sub> )P	246.8	162.6
GMPP(NH)P	188.4	131.4
GTP(γS)	4.3	7.8
GTP(γF)	281.4	
GTP(γMe)	320.8	214
GTP(γEt)	75.1	52.4
GTP(γPr)	55.8	24.6
GTP(γBu)	46.6	26.1
GTP(γEtNH <sub>2</sub> )	3.9	
GTP(γEtNHAc)	3.9	
GDP(βMe)	25	19.3
GDP(βPh)	7.1	19.3
ATP(γMe)	0	
ATP(γEtNHAc)	0	

<sup>a</sup> Nucleotide-dependent binding of EF-G to ribosomes was investigated by isolation of the nucleotide-EF-G-ribosome complexes either on Sephadex G-200 filtration or by ultracentrifugation (see Experimental Procedure). For filtration on Sephadex binding of EF-G to ribosomes was performed in a mixture containing in 0.020 ml: 50 mM Tris-HCl (pH 7.8), 20 mM MgCl<sub>2</sub>, 80 mM NH<sub>4</sub>Cl, 1 mM dithiothreitol, 250 pmol of ribosomes, 640 pmol of EF-G, and  $2-10^{-5}$  M nucleotides. Incubation time: 10 min at 30°. GTPase activity of 7 pmol of EF-G-ribosome complex passed through Sephadex G-200 was measured in a reaction mixture containing in 0.075 ml: 50 mM Tris-HCl (pH 7.8), 13 mM MgCl<sub>2</sub>, 80 mM NH<sub>4</sub>Cl, 1 mM dithiothreitol, 0.1  $\mu$ Ci of [<sup>32</sup>P] GTP (7 Ci/mol), and 200 pmol of 70S ribosomes. For ultracentrifugation binding mixture contained in 0.3 ml: 50 mM Tris-HCl (pH 7.8), 20 mM MgCl<sub>2</sub>, 80 mM NH<sub>4</sub>Cl, 1 mM dithiothreitol, 412 pmol of ribosomes, 960 pmol of EF-G,  $8 \times 10^{-5}$  M nucleotides. GTPase activity of the isolated ribosomes was measured as described for the EF-G-ribosome complex isolated on Sephadex G-200.

and the GMPP(CH<sub>2</sub>)P-dependent complexes, because observations indicate that they are functionally not equivalent (Modolell et al., 1973; Otaka and Kaji, 1974; Inoue-Yokosawa et al., 1974). The displacement was determined by measuring the amount of the radioactivity that remained bound to the EF-G-ribosome complexes in the presence of various concentrations of unlabeled nucleotide analogues. Figure 4A shows the results obtained with the fusidic acid-[<sup>3</sup>H]GDP-EF-G-ribosome complex. GDP was the most active compound in this reaction. At a concentration of  $2 \times 10^{-5}$  M, 50% of the bound [<sup>3</sup>H]GDP was displaced after 15 min of incubation. GTP(γS) was required in a somewhat higher concentration ( $5 \times 10^{-5}$  M). With increasing length of the side chain, the γ-phosphate esters turned out to be increasingly less effective. It is interesting to note that GMPP(CH<sub>2</sub>)P showed as little activity as GTP(γEtNHAc). The GDP analogues, GDP(βMe) and GDP(βPh), were the least effective compounds: they required concentrations of  $5 \times 10^{-2}$  M and  $10^{-2}$  M, respectively, to displace 50% of [<sup>3</sup>H]GDP.

With the [<sup>3</sup>H]GMPP(CH<sub>2</sub>)P-EF-G-ribosome complex (Figure 4B), GTPγF, GTP(γMe), and GMPP(CH<sub>2</sub>)P were most active. Under the conditions chosen 50% displacement occurred in a concentration range of  $1-2 \times 10^{-5}$  M. All triphosphate esters of GTP tested, as well as GTP(γF), were more effective in the displacement of [<sup>3</sup>H]GMPP(CH<sub>2</sub>)P than of [<sup>3</sup>H]GDP.

Additional presence of fusidic acid during formation of

the  $[^3\text{H}]\text{GMP}(\text{CH}_2)\text{-EF-G-ribosome}$  complex significantly affected the patterns of the reaction (Figure 4C).  $\text{GTP}(\gamma\text{S})$  and GDP became more effective than  $\text{GMPP}(\text{CH}_2)\text{P}$ , while  $\text{GTP}(\gamma\text{EtNHAc})$  became nearly as active as  $\text{GMPP}(\text{CH}_2)\text{P}$ . The diphosphate analogues  $\text{GDP}(\beta\text{Me})$  and  $\text{GDP}(\beta\text{Ph})$  showed little activity in all three systems.

#### Discussion

The introduction of different modifications at the terminal phosphate of GTP and GDP allowed us to obtain some general conclusions about the effect of such alterations on the interaction of the nucleotide with the active site of the ribosome-EF-G GTPase. Modification of the  $\gamma$ -phosphate results in compounds which, with the exception of  $\text{GTP}(\gamma\text{S})$ , cannot be hydrolyzed by EF-G and the ribosome. These analogues are, however, competitive inhibitors of GTP hydrolysis and thus are able to interact with its catalytic center. Since only one hydroxyl group in the terminal phosphate of our nucleotide analogues was modified, these results suggest that both negative charges of the two hydroxyl groups of the  $\gamma$ -phosphate are essential for compounds to act as substrates, but one is sufficient for the binding to the active site. One has, therefore, to conclude that the second negative charge of the  $\gamma$ -phosphate does not significantly contribute to the binding energy. The need of two negative charges in the terminal phosphate of GTP for hydrolysis seems to be strengthened by the observation that among the analogs only  $\text{GTP}(\gamma\text{S})$  can be hydrolyzed and be active in poly(U)-dependent poly(phenylalanine) incorporation even if at a lower rate than GTP.

A nucleophilic attack in an  $\text{S}_\text{N}2$  mechanism on the  $\gamma$  phosphorus has recently been proposed as a mechanism for GTP hydrolysis in ribosome-EF-G GTPase (Rohrbach et al., 1974). However, an alternative reaction, the formation of metaphosphate in an elimination mechanism, which is operative in the hydrolysis of phosphate monoesters (Benkovic and Schray, 1973) has also to be considered for this GTPase. The GTP esters might be expected still to be able to undergo an  $\text{S}_\text{N}2$ -type reaction, although one cannot exclude that the proper alignment of the functional groups necessary for the enzymatic reaction might be impaired by the ester group. If the hydrolysis of GTP, however, proceeds by the elimination mechanism one would expect the esters to be inert since there is no evidence for elimination of metaphosphate from diesters (Benkovic and Schray, 1973). The observed stability of GTP esters might therefore be an indication of an elimination type mechanism for this enzyme system. Because of the inherent difficulties in extrapolating from nonenzymatic to enzymatic reactions the possibility that the EF-G GTPase might follow an elimination rather than an  $\text{S}_\text{N}2$  mechanism cannot be more than a suggestion at the present time.

The rate of hydrolysis of  $\text{GTP}(\gamma\text{S})$  is unexpectedly high compared to that of GTP. The cleavage step of  $\text{ATP}(\gamma\text{S})$  in the myosin-catalyzed reaction is about 1000 times slower than that of ATP (Bagshaw et al., 1974). Also, the hydrolysis of  $\text{ATP}(\gamma\text{S})$  by alkaline phosphatase is extremely slow (Goody and Eckstein, 1971). On the other hand, the ATPase of the sarcoplasmic reticulum (Gratecos and Fischer, 1974) seems to cleave  $\text{ATP}(\gamma\text{S})$  faster than ATP. Whether these variations in relative rates are due to different mechanisms for different enzymes has to await further study. The activity in ribosome-EF-G-dependent reactions of the alkyl ester derivatives of GTP could be correlated to the length of the alcohol: increasing the number of carbon

atoms in the added side chain progressively decreased their inhibitory activity likely as a direct consequence of a reduced accessibility to the active site.

The same chemical modification in the terminal phosphate of GTP and GDP does not cause comparable inhibitory effects in the EF-G-dependent reactions:  $\text{GDP}(\beta\text{F})$  and  $\text{GDP}(\beta\text{Me})$  are about 100-fold less active than the corresponding GTP analogues. Since GDP can effectively compete with GTP for the active site, this indicates together with the results obtained with the GTP analogues that the presence of at least three negative charges in the phosphate chain of the nucleotide is needed for effective binding to the active site.

Since the actual hydrolysis of the  $\gamma$ -phosphate is preceded by the formation of a ternary complex with EF-G and ribosomes, it was to be expected that the activity of the different analogues in complex formation followed a pattern similar to their activity in the GTPase reaction. Substitution of one hydroxyl group of the  $\gamma$ -phosphate with a fluoride produced an effective GTP analogue, which could apparently enable all ribosomes to participate in the formation of the EF-G-ribosome complex. Thus the  $\text{GTP}(\gamma\text{F})$ -induced EF-G-ribosome complex would be most suitable for the identification of the ribosomal components neighboring EF-G by the use of cross-linking reagents in the absence of fusidic acid. A clear relationship between the action of fusidic acid and the structure of the different nucleotide analogues cannot be seen: only the aminoalkyl ester derivatives,  $\text{GTP}(\gamma\text{Ph})$ , as well as  $\text{GDP}(\beta\text{F})$  and  $\text{GDP}(\beta\text{Me})$ , are considerably stimulated by this antibiotic. The different properties of the various GTP and GDP analogues relative to their sensitivity toward fusidic acid is likely to be the result of a different functional alignment of the components in the ternary complexes. Structural modifications in the  $\beta$ - $\gamma$ -phosphate bond, as in  $\text{GMPP}(\text{NH})\text{P}$  and  $\text{GMPP}(\text{CH}_2)\text{P}$ , also cause different sensitivity toward fusidic acid. Although these two nucleotide analogues inhibited GTPase activity to a similar degree, only the ability of  $\text{GMPP}(\text{NH})\text{P}$  to stimulate complex formation was increased by fusidic acid. The observation that the spatial configuration of imidodiphosphate is closer to that of pyrophosphate than to methylene diphosphonate (Larsen et al., 1969) might explain the greater similarity of  $\text{GMPP}(\text{NH})\text{P}$  to GTP. The molar amounts of  $[^3\text{H}]\text{EF-G}$  and labeled GDP,  $\text{GTP}(\gamma\text{F})$ ,  $\text{GTP}(\gamma\text{S})$ , and  $\text{GMPP}(\text{CH}_2)\text{P}$  measured in the respective EF-G-ribosome complexes were equivalent. The possibility that two molecules of EF-G are required for each  $\text{GMPP}(\text{CH}_2)\text{P}$  bound to the ribosome as reported by Kuriki (1973) is not supported by our experiments. The displacement of GDP and  $\text{GMPP}(\text{CH}_2)\text{P}$  from their respective complexes with EF-G and ribosomes underlined the different properties of the nucleotide analogues and the difference between the  $\text{GMPP}(\text{CH}_2)\text{P}$ - and the GDP-fusidic acid-dependent complexes. In general, nucleotide analogues insensitive to fusidic acid, as  $\text{GTP}(\gamma\text{F})$  and  $\text{GTP}(\gamma\text{Me})$ , displaced  $\text{GMPP}(\text{CH}_2)\text{P}$  more readily than GDP. Nucleotide analogues sensitive to fusidic acid were most effective in the  $\text{GMPP}(\text{CH}_2)\text{P}$  system when fusidic acid was present, indicating that the antibiotic increased their stability relative to that of the  $\text{GMPP}(\text{CH}_2)\text{P}$ -dependent EF-G-ribosome complex. The possibility to improve the accessibility of a nucleotide analogue to the active site by displacing a compound prebound in the EF-G-ribosome complex was tempting. In this regard it was of particular interest that  $\text{GTP}(\gamma\text{EtNHAc})$ , a potential substrate for affinity labeling,

M. V. L. T. P. A. R. S.



which was a poor inhibitor of the EF-G GTPase activity, dramatically increased its activity in the displacement reaction by going from the GDP to the GMPP(CH<sub>2</sub>)P system in the presence of fusidic acid. Whether this increased activity corresponds to a specific binding to the EF-G-ribosome complex remains, however, to be proved.

The possibility of a covalent binding of bromo- or chloroacetyl aminoalkyl ester derivatives of GTP to the active site of the EF-G-ribosome is presently under investigation. GTP( $\gamma$ F) was unfortunately found to be unable to react covalently with EF-G or the ribosome. ATP( $\gamma$ F) could also not be bound irreversibly to myosin (Haley and Yount, 1972). Similarly, attempts to use a ribose-modified GDP analogue, GDP<sup>ox-red</sup>, as an affinity label for EF-G and ribosome were also not successful (Bodley and Gordon, 1974). GDP( $\beta$ Ph), the parent compound of the azidophenyl derivative of GDP, GDP( $\beta$ Azph), which has been used for photoaffinity labeling of the 50S ribosomal subunit (Maassen and Möller 1974), showed little activity in the EF-G-dependent reactions we tested.

Nucleoside triphosphate analogues modified in the  $\gamma$ -phosphate have hardly been studied in enzymatic reactions and it is therefore difficult to say whether they will be generally useful. ATP( $\gamma$ F) (Haley and Yount, 1972) and ATP( $\gamma$ Me) (R. S. Goody, personal communication) have been found to be very weak inhibitors of the myosin-dependent ATPase. Also for DNA-dependent RNA polymerase from *E. coli* ATP( $\gamma$ Me) and ATP( $\gamma$ F) are only very poor inhibitors at best (Sternbach and Armstrong, personal communication). It could well be that only a certain class of ATP- or GTP-utilizing enzymes can tolerate such modifications at the  $\gamma$ -phosphate without appreciable loss of affinity to the active site.

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# Interaction of Substrate Analogues with *Escherichia coli* DNA-Dependent RNA Polymerase

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The inhibition of RNA polymerase with ATP and UTP analogues modified in the phosphate and ribose moieties has been investigated.

1. Modification of the terminal phosphate with a loss of the negative charge [adenosine 5'-(3-*O*-methyl)triphosphate,  $K_i = 1.75$  mM] substantially weakens the binding ability of these analogues to the enzyme whereas modification with retention of the charge is not so detrimental [adenosine tetraphosphate,  $K_i = 0.17$  mM].

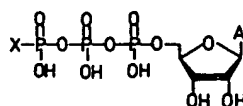
2. 2'-Modified analogues are only weak competitive inhibitors [2'-amino-2'-deoxyadenosine 5'-triphosphate,  $K_i = 2.3$  mM] of their corresponding substrates [ATP,  $K_m = 0.07$  mM] whereas 3'-modified analogues are extremely potent in their inhibition [3'-amino-3'-deoxyadenosine 5'-triphosphate,  $K_i = 2.3$   $\mu$ M].

3. A difference was observed in the inhibition of the elongation step of RNA polymerase by ATP and UTP analogues. Thus ATP analogues showed a strong binding to the  $C_T$  form of the poly[d(A-T)] ternary complex and only a weak binding to the  $C_A$  form. UTP analogues, on the other hand, showed a similar binding to both forms of the complex.

In studies directed towards the affinity labelling of DNA-dependent RNA polymerase we were interested in the effect of the chemical modification of substrates such as ATP and UTP in their affinity towards the enzyme. The design of an affinity label should be such that the derivative retains a strong affinity for the active site of the enzyme, and so it is important to determine those modifications of the normal substrates which do not greatly affect the binding ability. Having employed an analogue modified in the base [1], we wished to extend our research to the phosphate and ribose moieties. This work was prompted by the discovery that several guanosine triphosphates modified at the terminal phosphate are strong competitive inhibitors of the ribosomal GTPase [2]. Furthermore, it has recently been shown [3] that ATP- $\gamma$ -anilide, in which the terminal phosphate has been substituted with aniline, is a substrate for RNA polymerase although no details of the affinity were given.

We, therefore, chose to investigate several analogues modified at the terminal phosphate (I) and also

at the 2'- and 3'-hydroxyls of the ribose ring (II,III) with a view to future exploitation as affinity labels.

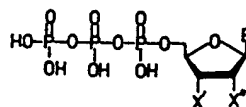


I A = Adenine

a) X = F

b) X = OCH<sub>3</sub>

c) X = OPhenyl



II B = Uracil

a) X = OH X' = NH<sub>2</sub>

b) X = OH X' = Cl

III B = Adenine

a) X = OH X' = NH<sub>2</sub>

b) X = NH<sub>2</sub> X' = OH

c) X'X'' =

The ability of the analogues to support poly[d(A-T)]-directed transcription by RNA polymerase was

Abbreviations. br<sup>3</sup> ATP, 8-bromo-adenosine 5'-triphosphate; 3'-ATP, 3'-deoxyadenosine 5'-triphosphate.

Enzyme. DNA-dependent RNA polymerase (EC 2.7.7.6).

studied as well as their inhibition of this process. In the case of the inhibition studies the normal kinetic assay was employed which includes chain initiation, elongation and termination.

The inhibition of the chain elongation process of RNA synthesis using the assay devised by Rhodes and Chamberlin [4] was also investigated with several analogues.

## MATERIALS AND METHODS

### Materials

ATP, UTP,  $\text{br}^8\text{ATP}$  and rifampicin were purchased from Boehringer. Adenosine tetraphosphate was a product of Sigma Chemical company and was purified from contaminating ATP by chromatography over DEAE-Sephadex A-25.  $[^{14}\text{C}]\text{ATP}$ ,  $[^{14}\text{C}]\text{UTP}$  and  $[^3\text{H}]\text{UTP}$  were supplied by Schwarz and  $[\alpha\text{-}^{32}\text{P}]\text{ATP}$  by New England Nuclear. Poly[d(A-T)] was obtained from Miles Laboratories Inc. For the preparation of the ternary complex a sample of poly[d(A-T)] kindly supplied by Dr T. M. Jovin was employed.

### Synthetic Nucleotides

The  $\gamma$ -phosphate-modified ATP analogues adenosine 5'-(3-fluoro)triphosphate (Ia), adenosine 5'-(3-O-methyl)triphosphate (Ib) and adenosine 5'-(3-O-phenyl)triphosphate (Ic) were synthesized according to the procedure described for the corresponding GTP analogues [2]. The analogues were stable against alkaline phosphatase, and so to eliminate contamination by ATP they were treated with this enzyme prior to use, and the nucleotide was recovered by chromatography on DEAE-Sephadex A-25. Uridine tetraphosphate was prepared from UTP and inorganic phosphate by a conventional procedure [5], 2'-amino-2'-deoxyuridine 5'-triphosphate (IIa), 2'-chloro-2'-deoxyuridine 5'-triphosphate (IIb), 2'-amino-2'-deoxyadenosine 5'-triphosphate (IIIa), 2',3'-epoxy-2',3'-dideoxyadenosine 5'-triphosphate (IIIc) were kind gifts of Dr J. B. Hobbs and 3'-amino-3'-deoxyadenosine 5'-triphosphate (IIId) was kindly donated by Dr M. Sprinzl.

### Enzyme Purification and Assay

RNA polymerase holo enzyme was a generous gift of Dr H. Sternbach, and had been purified by a described procedure [6]. The enzyme was greater than 95% pure according to dodecylsulfate gel electrophoresis. It was assayed in an incubation mixture containing 40 mM Tris-HCl, pH 8.0, 10 mM  $\text{MgCl}_2$ , 5 mM dithioerythritol, 50 mM KCl, and template, enzyme and substrates as described in the legends to

the figures. For the kinetic studies the reaction was started by the addition of the enzyme and after incubation of the solution at 37 °C for 5 min a 50- $\mu\text{l}$  aliquot was removed and the amount of label incorporated into acid-insoluble material was determined by a published procedure [7].

### Preparation of the Poly[d(A-T)] Complex and Assay

The ternary complex was prepared and assayed exactly according to the procedure of Rhodes and Chamberlin [4]. The assay mixture (0.25 ml) contained 40 mM KCl, 10 mM  $\text{MgCl}_2$ , 10 mM mercaptoethanol and 10  $\mu\text{g/ml}$  rifampicin. For the kinetic studies the reaction was terminated after a 5-min incubation at 37 °C.

## RESULTS AND DISCUSSION

### Ability of the Analogues to Support Transcription of Poly[d(A-T)]

In accordance with previous results [3] the  $\gamma$ -modified ATP analogues Ia, Ib and Ic were able to support poly[d(A-T)]-directed incorporation of  $[^{14}\text{C}]\text{UMP}$  into acid-insoluble material in place of ATP (Fig. 1). The rate of incorporation, however, was much lower than that in the presence of ATP. Adenosine tetraphosphate as well as the uridine analogue (Table I) showed only marginal incorporation which was about 1% that with the triphosphate. This may represent slight contamination of the tetraphosphates with the corresponding triphosphate rather than genuine incorporation. In the case of adenosine tetraphosphate this lack of incorporation is presumably caused by the inability of the tetraphosphate to function in chain elongation since it is able to support the pyrophosphate exchange reaction [8] and, therefore, chain initiation. The  $\gamma$ -modified phosphate analogues were as effective as the tetraphosphate in the pyrophosphate exchange reaction (Armstrong and Eckstein, unpublished results).

The 2'- and 3'-modified nucleotides showed little incorporation above that of the background and so they are at best poor substrates for RNA polymerase. It is possible that they act as chain terminators as is the case for 2'-O-methyladenosine 5'-triphosphate [10] and 3'-dATP [9] but this was not investigated.

However, poor substrate ability does not necessarily imply that the analogues bind weakly to the active site since poor incorporation may only reflect a slower reaction rate due to the chemical modification. Since we were primarily interested in the affinity of these compounds for the active site we, therefore, tested their ability to inhibit poly[d(A-T)]-directed transcription.



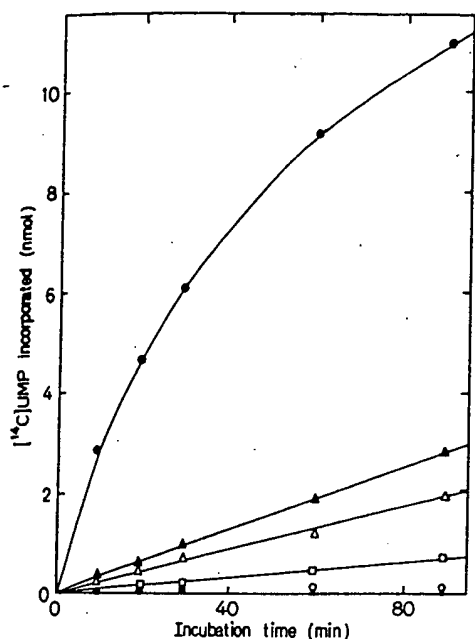


Fig. 1. Ability of the ATP analogues to support poly[d(A-T)] transcription. The incubation mixture (0.2 ml) was as described in Materials and Methods and contained in addition 0.2  $A_{260}$  unit poly[d(A-T)], 1 mM [ $^{14}\text{C}$ ]UTP (spec. act. 2300 counts  $\text{min}^{-1}$   $\text{nmol}^{-1}$ ), 8.4  $\mu\text{g}$  enzyme and (●) 1 mM ATP, (▲) 1 mM Ib, (Δ) 1 mM Ic, (□) 1 mM adenosine tetraphosphate or (×)  $\text{H}_2\text{O}$ . The mixture was incubated at 37°C and 20- $\mu\text{l}$  aliquots were assayed after various times as described in Materials and Methods. 1 mM 2'-amino-2'-deoxyadenosine 5'-triphosphate gave similar incorporation data to adenosine tetraphosphate.

Table 1. Transcription of poly[d(A-T)] with the UTP analogues. The incubation mixture (0.1 ml) was as described in Materials and Methods and contained in addition 0.2  $A_{260}$  unit poly[d(A-T)], 1 mM [ $^{14}\text{C}$ ]ATP (spec. act. 1850 counts  $\text{min}^{-1}$   $\text{nmol}^{-1}$ ), 8.4  $\mu\text{g}$  enzyme and the substrate as given in the table. The mixture was incubated at 37°C and 15- $\mu\text{l}$  aliquots were assayed as described in Materials and Methods.

Substrate	[ $^{14}\text{C}$ ]AMP incorporated in	
	10 min	120 min
	nmol	
1 mM UTP	2.02	9.38
1 mM uridine tetraphosphate	0.049	0.156
1 mM 2'-amino-2'-deoxyuridine 5'-triphosphate (IIa)	0.064	0.199
1 mM 2'-chloro-2'-deoxyuridine 5'-triphosphate (IIb)	0.036	0.050
-	0.038	0.070

#### Inhibition of Poly[d(A-T)] Transcription

The  $\gamma$ -phosphate-modified ATP analogues were competitive inhibitors of ATP, but this inhibition was weak (Table 2), the  $K_i$  values being 10–20 times greater than the  $K_m$  for ATP. Thus the poor sub-

Table 2. Inhibition of poly[d(A-T)] transcription by ATP analogues. The incubation mixture (0.1 ml) was as described in Materials and Methods and contained in addition 0.2  $A_{260}$  unit poly[d(A-T)], 0.4 mM UTP, 1.3  $\mu\text{g}$  enzyme, varying concentrations of [ $^{14}\text{C}$ ]ATP (spec. act. 18000 counts  $\text{min}^{-1}$   $\text{nmol}^{-1}$ ) and a fixed concentration of inhibitor. The initial rate was determined as described in Materials and Methods and the  $K_i$  for the analogue was then determined from a Lineweaver-Burk plot. The inhibition by all the analogues tested was competitive.

Substrate	$K_m$	Inhibitor	$K_i$
	mM		mM
ATP	0.07	adenosine 5'-(3-fluoro)-triphosphate (Ia)	0.74
		adenosine 5'-(3-O-methyl)triphosphate (Ib)	1.72
		adenosine 5'-(3-O-phenyl)triphosphate (Ic)	1.8
		adenosine tetraphosphate	0.17
		2',3'-epoxy-2',3'-dideoxyadenosine 5'-triphosphate (IIIc)	2.12
		2'-amino-2'-deoxyadenosine 5'-triphosphate (IIIa)	2.3
		3'-amino-3'-deoxyadenosine 5'-triphosphate (IIIb)	0.0023

Table 3. Inhibition of poly[d(A-T)] transcription by the UTP analogues.

The procedure was as in the legend to Table 2 except that a fixed ATP concentration of 0.4 mM was employed and the [ $^{14}\text{C}$ ]UTP concentration was varied in the presence of a fixed concentration of inhibitor. The inhibition by all the analogues tested was competitive.

Substrate	$K_m$	Inhibitor	$K_i$
	mM		mM
UTP	0.064	2'-amino-2'-deoxyuridine 5'-triphosphate (IIa)	0.73
		2'-chloro-2'-deoxyuridine 5'-triphosphate (IIb)	0.75
		uridine tetraphosphate	0.28

strate ability of these compounds can be partly attributed to their high dissociation constants. In contrast, adenosine tetraphosphate was a much more potent inhibitor of ATP, as was uridine tetraphosphate of UTP (Table 3). It appears, therefore, that a modification of the terminal phosphate which results in the loss of a negative charge substantially reduces the binding ability of these derivatives, whereas a modification with retention of the charge is not so detrimental.

The 2'-modified UTP analogues were relatively weak competitive inhibitors of UTP (Table 3) and 2',3'-epoxy-2',3'-dideoxyadenosine 5'-triphosphate and 2'-amino-2'-deoxyadenosine 5'-triphosphate were even weaker competitive inhibitors of ATP (Table 2).

In contrast 3'-amino-3'-deoxyadenosine 5'-triphosphate was an extremely powerful inhibitor of ATP (Table 2) its  $K_i$  being almost 20 times lower than the  $K_m$  for ATP and a factor of  $10^3$  smaller than the  $K_i$  for the 2'-modified analogue. This data is similar to that found for the inhibition of RNA polymerase from *Pseudomonas putida* [11] with 3'-dATP where a  $K_i$  value of  $2 \mu\text{M}$  was reported compared to a  $K_m$  value for ATP of  $60 \mu\text{M}$ . On the other hand 2'-dATP is a poor substrate for RNA polymerase [12-15] and appears to be only a weak inhibitor of ATP incorporation [16]. These results with the 2'- and 3'-modified nucleoside triphosphates indicate that the 2'-hydroxyl although not essential is, nevertheless, necessary for efficient binding of the triphosphate to the active site of the enzyme. The 3'-hydroxyl, however, does not appear to be important for the binding process, but is obviously required for phosphodiester bond formation.

#### Inhibition of Chain Elongation

The inhibition of the elongation reaction can be studied independently of initiation and termination using the assay devised by Rhodes and Chamberlin [4]. The authors studied the inhibition of this step with substrate and non-complementary nucleoside triphosphates. We have extended this to include complementary nucleoside triphosphate analogues. For the poly[d(A-T)] ternary complex two states were postulated,  $C_T$  and  $C_A$ , the former being able to incorporate an AMP residue into the growing RNA chain, and the latter a UMP residue. Both forms can potentially interact with inhibitors and the full rate equation for this inhibition as was derived by Rhodes and Chamberlin is

$$\frac{V}{v} = 1 + \left(\frac{K_A}{A}\right) \left(1 + \frac{I}{K_{IA}^I}\right) + \left(\frac{K_U}{U}\right) \left(1 + \frac{I}{K_{IU}^I}\right) \quad (1)$$

where  $v$  is the initial velocity of the reaction at concentrations of MgATP and MgUTP of  $A$  and  $U$  respectively;  $V$  is the maximal velocity;  $K_A$  and  $K_U$  are the Michaelis constants for each substrate;  $I$  is the inhibitor concentration and  $K_{IA}^I$  and  $K_{IU}^I$  are the inhibition constants for inhibition of the  $C_T$  and  $C_A$  forms of the ternary complex respectively.

In order to determine  $K_{IA}^I$  and  $K_{IU}^I$  the proper experimental conditions should be selected. Thus, to measure  $K_{IA}^I$  one need only take a concentration of UTP much greater than  $K_{IU}^I$  such that the rate equation simplifies to

$$\frac{V}{v} = 1 + \left(\frac{K_A}{A}\right) \left(1 + \frac{I}{K_{IA}^I}\right) \quad (2)$$

Simple competitive inhibition would then be expected.

Employing a high concentration of UTP, and varying the concentration of ATP, adenosine tetra-

phosphate proved to be a competitive inhibitor of ATP (Fig. 2A), with a  $K_{IA}$  of  $52 \mu\text{M}$  ( $K_A = 5.3 \mu\text{M}$ ). However, when the inhibition of UTP incorporation by adenosine tetraphosphate was studied at high ATP concentration uncompetitive inhibition was observed (Fig. 2B). This can be explained by a disparity between the two inhibition constants  $K_{IA}^I$  and  $K_{IU}^I$ . If  $K_{IA}^I$  is much smaller than  $K_{IU}^I$  then the term  $(K_A/A) [1 + (I/K_{IA}^I)]$  can no longer be neglected and a mixed type of inhibition would be expected which would tend towards uncompetitive inhibition as the difference between the two inhibition constants increased. Thus adenosine tetraphosphate binds much more weakly to the  $C_A$  form of the ternary complex than to the  $C_T$  form. This is in agreement with the model proposed for chain elongation [4, 17, 18] in which triphosphates bind weakly to the elongation site, followed by a transformation to a much stronger binding in the case of the correct triphosphate. Adenosine tetraphosphate is, therefore, able to induce strong complex formation to the  $C_T$  complex, but is only able to bind weakly to the  $C_A$  complex. Similar results were obtained using  $\text{br}^8\text{ATP}$  a nucleotide which adopts the *syn* conformation. This analogue was a competitive inhibitor of ATP with a  $K_{IA}$  of  $6 \mu\text{M}$ , whereas uncompetitive inhibition was observed against UTP. It can thus induce strong complex formation with the  $C_T$  complex. Kapuler and Reich [19] have previously shown that 8-substituted GTP analogues are strong competitive inhibitors of GTP.

In contrast to the ATP analogues uridine tetraphosphate was a competitive inhibitor of both UTP (Fig. 3A) and ATP (Fig. 3B) the inhibition constants being  $0.182$  and  $0.307 \text{ mM}$  respectively ( $K_U = 13 \mu\text{M}$ ). Thus it shows a similar binding ability to both the  $C_A$  and  $C_T$  forms of the ternary complex. The  $K_{IA}$  value of  $0.307 \text{ mM}$  is substantially lower than the corresponding value for UTP of  $1.81 \text{ mM}$  [4] and it therefore appears that uridine tetraphosphate can induce strong complex formation to the  $C_T$  ternary complex whereas UTP shows only the weak triphosphate binding. A second UTP analogue, 2'-amino-2'-deoxy-uridine 5'-triphosphate was also a competitive inhibitor of both UTP and ATP, the values for  $K_{IA}$  and  $K_{IA}$  being  $0.33 \text{ mM}$  and  $0.65 \text{ mM}$  respectively.

#### CONCLUSION

The inhibition data with the substrate analogues give some indication of the factors which are important for the binding of substrates to RNA polymerase. With regard to the affinity labelling of the enzyme by modification of the phosphate or ribose residues of the substrate, the 3'-position of the ribose ring appears to offer the best possibility for modification without reducing the affinity of the analogue. We are, therefore, investigating this further.

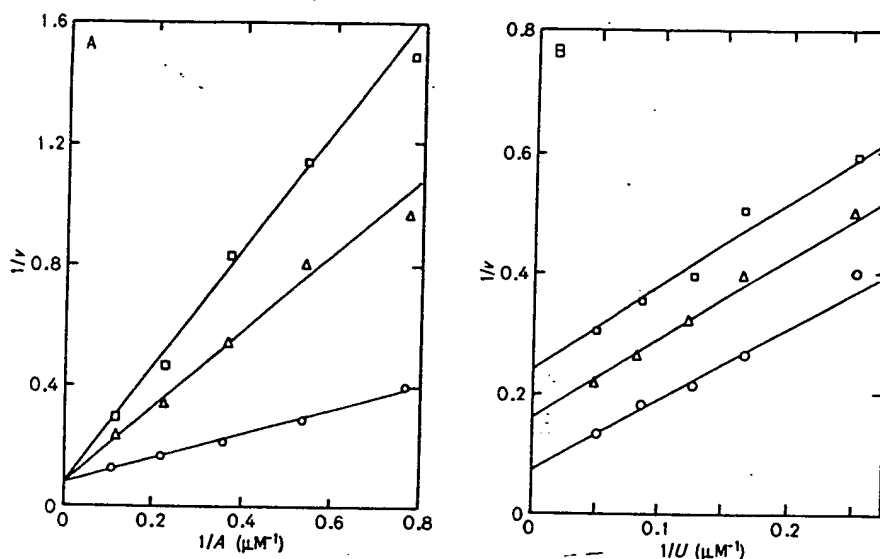


Fig. 2. Inhibition of chain elongation by adenosine tetraphosphate. (A) The UTP concentration was held constant at 0.2 mM and the  $[\alpha\text{-}^{32}\text{P}]\text{ATP}$  (spec. act.  $1400 \text{ counts min}^{-1} \text{ pmol}^{-1}$ ) concentration was varied in the presence of the following inhibitor concentrations: (O) 0, ( $\Delta$ ) 0.185 mM, ( $\square$ ) 0.37 mM. (B) The ATP concentration was held constant at 0.1 mM and the  $[\text{H}]\text{UTP}$  (spec. act.  $1000 \text{ counts min}^{-1} \text{ pmol}^{-1}$ ) concentration was varied in the presence of the following inhibitor concentrations: (O) 0, ( $\Delta$ ) 1.0 mM, ( $\square$ ) 2.0 mM.  $v$  was measured as pmol labelled nucleotide incorporated in 5 min

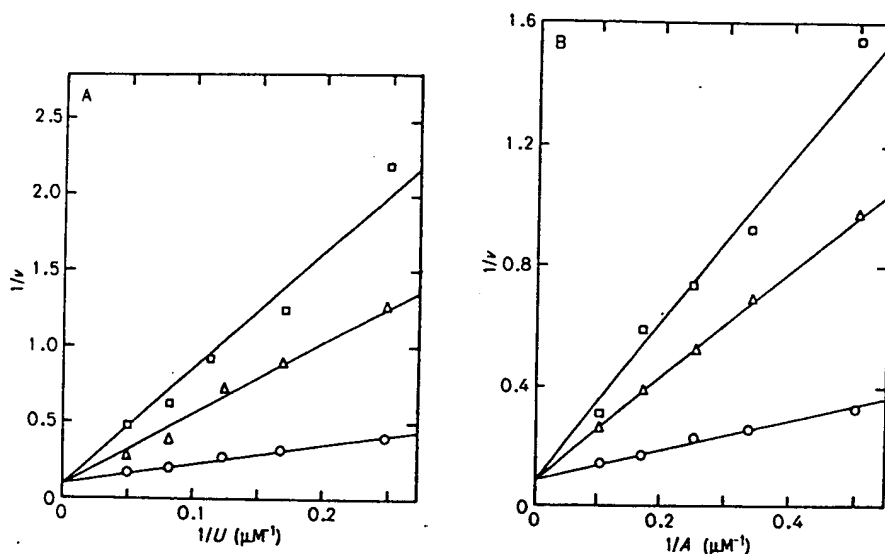


Fig. 3. Inhibition of chain elongation by uridine tetraphosphate. (A) The ATP concentration was held constant at 0.1 mM and the  $[\text{H}]\text{UTP}$  concentration was varied in the presence of the following inhibitor concentrations: (O) 0, ( $\Delta$ ) 0.4 mM, ( $\square$ ) 0.8 mM. (B) The UTP concentration was held constant at 0.1 mM, and the  $[\alpha\text{-}^{32}\text{P}]\text{ATP}$  concentration was varied in the presence of the following inhibitor concentrations: (O) 0, ( $\Delta$ ) 1.5 mM, ( $\square$ ) 3.0 mM.  $v$  was measured as pmol labelled nucleotide incorporated in 5 min

The inhibition studies with the ternary complex indicate that UTP analogues in contrast to ATP are able to induce strong complex formation to both the  $C_A$  and  $C_T$  forms of the poly[d(A-T)] ternary complex. In view of the potent inhibition of RNA polymerase by 3'-modified ATP's such compounds along with their corresponding UTP derivatives should be useful for further investigation of the binding of substrates and their analogues to the ternary complex.

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## Nonionic Nucleic Acid Analogues. Synthesis and Characterization of Dideoxyribonucleoside Methylphosphonates<sup>†</sup>

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**ABSTRACT:** A series of dideoxyribonucleoside methylphosphonate analogues, dNpN and dNpNp, which contain a nonionic 3'-5' methylphosphonyl internucleoside linkage were prepared. The two diastereoisomers, designated isomers 1 and 2, of each dimer differ in configuration of the methylphosphonate group and were separated by column chromatography. The diastereoisomers of each dimer have different conformations in solution as shown by ultraviolet hypochromicity data and their circular dichroism spectra. For example, dApA isomer 1 is more highly stacked than isomer 2, although both isomers are less stacked than the dinucleoside monophosphate, dApA. The circular dichroism spectrum of isomer 1 is very similar to that of dApA, while the CD spectrum of isomer 2 shows a loss of molecular ellipticity,  $[\theta]$ , at 270 nm and a greatly diminished  $[\theta]$  at 250 nm. These results suggest that the stacked bases of dApA isomer 1 tend to orient in an oblique manner, while those in isomer 2 tend to orient in a parallel manner. This interpretation is verified by the <sup>1</sup>H

NMR study of these dimers (L. S. Kan, D. M. Cheng, P. S. Miller, J. Yano, and P. O. P. Ts'o, unpublished experiments). Both diastereoisomers of dApA form 2U:1A and 2T:1A complexes with poly(U) and poly(dT), respectively. The higher  $T_m$  ( $T_m$  of poly(U)-isomer 1, 15.4 °C;  $T_m$  of poly(U)-isomer 2, 19.8 °C;  $T_m$  of poly(dT)-isomer 1, 18.7 °C;  $T_m$  of poly(dT)-isomer 2, 18.4 °C) values of these complexes vs. those of the corresponding dApA-polynucleotide complexes ( $T_m$  of poly(U)-dApA, 7.0 °C;  $T_m$  of poly(dT)-dApA, 9.2 °C) result from decreased charge repulsion between the nonionic dimer backbone and the negatively charged polymer backbone. The difference in conformations between dApA isomer 1 and dApA isomer 2 is reflected in the  $T_m$  of the isomer 1-poly(U) complex which is 4.4 °C lower than that of the isomer 2-poly(U) complex. Since these nonionic oligonucleotide analogues are taken up by cells in culture, they show promise as molecular probes for the function and structure of nucleic acids inside living cells.

**S**tudies on nucleic acid analogues and derivatives possessing modified internucleoside linkages have made important contributions to understanding nucleic acid conformation in solution and have provided materials for various biochemical and biological studies (Jones et al., 1970, 1973; Pitha et al., 1971; Letsinger et al., 1976; Blob et al., 1977; Mungall & Kaiser, 1977; Vosberg & Eckstein, 1977). In a series of seven papers, our laboratory has reported studies on the physical, biochemical, and biological properties of one class of nonionic nucleic acid derivative, the oligonucleotide alkyl phosphotriesters. The physical properties of dinucleotide methyl and ethyl phosphotriesters have been studied by ultraviolet, circular dichroism, infrared, and proton nuclear magnetic resonance spectroscopy (Miller et al., 1971; Deboer et al., 1973). The interaction of deoxyribooligonucleotide ethyl phosphotriesters with sequences complementary to the amino acid accepting stem and antico-

don region of transfer RNA has been characterized (Miller et al., 1974), and their inhibitory effects on in vitro aminoacylation have been studied (Barrett et al., 1974). More recently, the inhibitory effect of a 2'-O-methyl ribooligonucleotide triester,  $G_p^m(Et)G_p^m(Et)U$ ,<sup>1</sup> on cellular protein synthesis and growth of mammalian cells in culture has been reported (Miller et al., 1977). In addition, selective binding of an octathymidylate ethyl phosphotriester,  $[Tp(Et)]_8T$ , to poly(deoxyadenylic acid) has been extensively investigated (Pless & Ts'o, 1977).

In this and subsequent papers in this series (Kan et al., unpublished experiments; Cheng et al., unpublished experiments), we describe the synthesis, the interaction with com-

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<sup>1</sup> Abbreviations used: Np(Et)N, an oligonucleotide ethyl phosphotriester; dNpN, a deoxyribonucleotide dimer analogue containing a 3'-5' internucleoside methylphosphonate linkage (in this abbreviation the italic p represents the methylphosphonate linkage); MST, mesitylenesulfonyl tetrazolide; TPSCI, 2,4,6-triisopropylbenzenesulfonyl chloride; DCC, dicyclohexylcarbodiimide. The symbols used to represent protected nucleosides and dideoxyribonucleoside methylphosphonates follow the IU-PAC-IUB Commission on Biochemical Nomenclature recommendations (1970).

plementary polynucleotides, and the general conformational features of a novel type of nonionic dideoxyribonucleotide analogue, the dideoxyribonucleoside methylphosphonates. In these analogues, the natural phosphodiester linkage is replaced by an isosteric 3'-5'-linked methylphosphonate group. In terms of size, substitution of a methyl residue for the nonesterified oxygen of the phosphate group represents the smallest possible structural change which can be made in the nucleic acid backbone and which results in the removal of the electrostatic charge while still retaining the geometrical features of the phosphodiester linkage. Thus, this modification was expected to have only a relatively small steric perturbation on the conformation of the dimer in solution and on the interaction of the dimer with complementary polynucleotides.

Our synthetic procedure resulted in the separation of the two diastereoisomers of each dimer analogue. This synthetic scheme also allowed the preparation of analogues containing a  $^{13}\text{C}$ -enriched phosphonate methyl group. The influence of backbone configuration on overall dimer conformation was studied by ultraviolet, circular dichroism, and  $^1\text{H}$ ,  $^{13}\text{C}$ , and  $^{31}\text{P}$  nuclear magnetic resonance techniques, and the results were compared to the conformations of their parent dideoxyribonucleoside monophosphates. Furthermore, the effects of backbone configuration and the removal of the negative charge on the interaction of deoxyadenosine-containing dimers with poly(uridylic acid) and poly(thymidylic acid) were assessed.

#### Materials and Methods

Thymidine and 2'-deoxyadenosine were purchased from P-L Biochemicals and were checked for purity by paper chromatography before use. 5'-(*p*-Methoxytrityl)thymidine, 5'-(di-*p*-methoxytrityl)-*N*-benzoyldeoxyadenosine (Schaller et al., 1963), 3'-*O*-acetylthymidine, and 3'-*O*-acetyl-*N*-benzoyldeoxyadenosine (Zorbach & Tipson, 1968) were prepared according to published procedures. Diethyl [ $^{13}\text{C}$ ]methylphosphonate was prepared by reaction of [ $^{13}\text{C}$ ]methyl iodide (96% enriched) with triethyl phosphate followed by vacuum distillation of the product (bp 64–66 °C/2 mmHg) according to the procedure of Ford-Moore & Williams (1947). The pyridinium salt of methylphosphonic acid was prepared by hydrolysis of dimethyl methylphosphonate (K & K Laboratories) or diethyl [ $^{13}\text{C}$ ]methylphosphonate in 4 N hydrochloric acid, followed by isolation of the product as the barium salt (Höly, 1967). The barium salt was converted to the pyridinium salt by passage through a Dowex 50X pyridinium ion-exchange column. Mesitylenesulfonyl chloride (Aldrich Chemical Co.) was treated with activated charcoal and recrystallized from pentane immediately before use. 1*H*-Tetrazole was prepared by the method of Henry & Finnegan (1954). All solvents and reagents were purified as previously described (Miller et al., 1974).

Silica gel column chromatography was performed by using Baker 3405 silica gel (60–200 mesh). Thin-layer silica gel chromatography and thin-layer cellulose chromatography were done on plates manufactured by E. Merck Co. Paper chromatography was carried out on Whatmann 3MM paper using the following solvent systems: solvent A, 2-propanol-concentrated ammonium hydroxide-water (7:1:2 v/v); solvent C, 1 M ammonium acetate–95% ethanol (3:7 v/v); solvent F, 1-propanol-concentrated ammonium hydroxide-water (50:10:35 v/v); solvent I, 2-propanol-water (7:3 v/v). High-pressure liquid chromatography (LC) was performed on a Laboratory Data Control instrument using columns (2.1 mm  $\times$  1 m) packed with Du Pont Permaphase ODS reverse-phase material. Linear gradients (40 mL) from 0 to 75% methanol in water were used at a flow rate of 1 mL/min.

The LC mobility refers to the percentage of methanol in water required to elute the compound from the column.

For reactions carried out in pyridine, the reactants were dried by repeated evaporation with anhydrous pyridine and were then dissolved in anhydrous pyridine. Unless otherwise noted, all reactions and operations were performed at room temperature.

**Preparation of Mesitylenesulfonyl Tetrazolide.** Although the preparation of MST has been published (Stawinski et al., 1977), we followed a modified procedure originally suggested to us by Letsinger and co-workers (R. L. Letsinger, personal communication). A solution of 1*H*-tetrazole (3.36 g, 48 mmol) in 40 mL of dry methylene chloride containing 5.6 mL (40 mmol) of triethylamine was added dropwise to 40 mL of anhydrous ether containing mesitylenesulfonyl chloride (8.76 g, 40 mmol) at room temperature. After 2 h, the precipitated triethylammonium chloride was removed by filtration and washed with 50 mL of methylene chloride-ethyl ether (1:1 v/v). The filtrate was cooled to 0 °C and pentane was added until the solution became cloudy. This procedure was repeated until a total volume of 25 mL had been added over a 4-h period. After storage overnight at 4 °C, the resulting white solid was removed by filtration on a sintered glass filter. The solid was dissolved in 500 mL of anhydrous ethyl ether. The solution was then filtered to remove a small amount of insoluble material. The filtrate was evaporated to dryness, and the resulting mesitylenesulfonyl tetrazolide (4.46 g) was obtained in 44% yield. The MST was pure as indicated by silica gel TLC,  $R_f$  ( $\text{C}_6\text{H}_6$ ) 0.11 (mp 109–110 °C), and was stored in a desiccator at –80 °C. Under these storage conditions, the MST maintained its condensing activity for at least 1 month.

**Preparation of (MeOTr)TpCE.** (MeOTr)T (10.3 g, 20 mmol), the pyridinium salt of methylphosphonic acid (40 mmol), and Dowex pyridinium resin (0.5 g) were treated with dicyclohexylcarbodiimide (41.2 g, 200 mmol) in 100 mL of pyridine at 37 °C for 3 days. The resulting (MeOTr)Tp,  $R_f$  (silica gel TLC) 0.00 (EtOAc-THF, 1:1), appeared to be formed in approximately 86% yield as determined by LC. The material is eluted from the LC column with 37% methanol-water. Hydracrylonitrile (100 mL) was added to the reaction mixture, which was kept at 37 °C for 2 days. Aqueous pyridine (200 mL) was then added and the resulting dicyclohexylurea was removed by filtration. The filtrate was evaporated and dissolved in 250 mL of ethyl acetate, and the solution was extracted with three (250-mL) portions of water. The ethyl acetate solution was dried over anhydrous sodium sulfate. After filtration and evaporation, the mixture was chromatographed on a silica gel column (5.4  $\times$  37 cm) which was eluted with ether (1 L), ethyl acetate (1.2 L), and tetrahydrofuran (1.6 L). Pure (MeOTr)TpCE (7.5 g) was isolated in 55% yield after precipitation from tetrahydrofuran by addition of hexane. The monomer has  $R_f$  values of 0.32 (EtOAc-THF, 1:1) and 0.66 (20% MeOH- $\text{CHCl}_3$ ) on silica gel TLC and is eluted from the LC column with 54% methanol-water. The UV spectrum gave  $\lambda_{\text{max}}$  267 and 230 nm (sh) and  $\lambda_{\text{min}}$  250 nm ( $\epsilon_{260}/\epsilon_{280}$  1.44) in 95% ethanol.

**Preparation of (MeOTr)Tp Pyridinium Salt.** (MeOTr)TpCE (2.17 g, 3.36 mmol) was treated with 16.8 mL of 1 N sodium hydroxide in a solution containing 126 mL of dioxane and 25 mL of water for 15 min. The solution was neutralized by addition of Dowex 50X pyridinium resin. After filtration, the solution was evaporated and the residue was rendered anhydrous by evaporation with pyridine. (MeOTr)Tp (1.90 g, 2.83 mmol) was obtained in 84% yield after precipitation from pyridine by addition to anhydrous ether. The material

Table I: Preparation of Protected Dideoxyribonucleoside Methylphosphonates

monomers (mmol)	condensing agent <sup>a</sup> (mmol)	reaction time	dimer (mmol)	yield (%)
d(MeOTr)Tp (0.20) + dTOAc (0.22)	DCC (0.73)	3 days, 37 °C	d(MeOTr)TpTOAc (0.031)	16
d(MeOTr)Tp (2.40) + dTpCE (3.60)	MST (9.60)	3 h, rt <sup>b</sup>	d(MeOTr)TpTpCE (1.32)	55
d[(MeO) <sub>2</sub> Tr]bzAp (1.26) + d(bz)AOAc (1.50)	TPSCI (2.0)	4 days, 37 °C	d[(MeO) <sub>2</sub> Tr]bzApbzAOAc (0.50)	39
d[(MeO) <sub>2</sub> Tr]bzA (0.70) [ <sup>13</sup> C] <sub>p</sub> + d(bz)AOAc (1.05)	MST (2.8)	4 h, rt	d[(MeO) <sub>2</sub> Tr]bzA[ <sup>13</sup> C] <sub>p</sub> bzAOAc (0.29)	41
d[(MeO) <sub>2</sub> Tr]bzAp (0.85) + d(bz)ApCE (1.28)	MST (4.0)	6 h, rt	d[(MeO) <sub>2</sub> Tr]bzApbzApCE (0.39)	46
d(MeOTr)Tp (1.0) + d(bz)AOAc (1.0)	TPSCI (3.0)	16 h, 37 °C	d(MeOTr)TpApbzAOAc (0.35)	35
d[(MeO) <sub>2</sub> Tr]bzAp (1.0) + dTOAc (1.3)	TPSCI (1.5)	46 h, 37 °C	d[(MeO) <sub>2</sub> Tr]bzApTOAc (0.38)	38

<sup>a</sup> DCC, dicyclohexylcarbodiimide; TPSCI, triisopropylbenzenesulfonyl chloride; MST, mesitylenesulfonyl tetrazolide. <sup>b</sup> rt, room temperature.

has  $R_f$  values of 0.00 (EtOAc-THF, 1:1) and 0.04 (20% MeOH-CHCl<sub>3</sub>) on silica gel TLC. The UV spectrum gave  $\lambda_{\max}$  267 and 230 nm (sh) and  $\lambda_{\min}$  250 nm ( $\epsilon_{230}/\epsilon_{267}$  1.68;  $\epsilon_{260}/\epsilon_{280}$  1.44) in 95% ethanol.

The monomethoxytrityl group was removed from 70 mg (0.1 mmol) of d(MeOTr)Tp by treatment with 80% aqueous acetic acid. The resulting Tp (874  $A_{267}$  units, 0.095 mmol) was isolated in 95% yield by chromatography on a DEAE-Sephadex A-25 column (3 × 8.5 cm) using a linear gradient of ammonium bicarbonate (0.01–0.20 M, 500 mL). The monomer has the following  $R_f$  values on cellulose TLC: 0.41 (solvent A), 0.77 (solvent C), and 0.69 (solvent F). The UV spectrum gave  $\lambda_{\max}$  267 nm and  $\lambda_{\min}$  235 nm in water, pH 7.0. The <sup>1</sup>H NMR spectrum was consistent with the structure of the monomer (Kan et al., unpublished results).

**Preparation of TpCE.** (MeOTr)TpCE (3.26 g, 5.04 mmol) dissolved in 20 mL of methanol was treated with 80 mL of 80% acetic acid solution for 5 h at 37 °C. The solvents were removed by evaporation, and the residue was evaporated repeatedly with 50% toluene-tetrahydrofuran to remove the acetic acid. TpCE (1.80 g, 4.8 mmol) was obtained in 96% yield after precipitation from tetrahydrofuran (10 mL) by addition of hexane (200 mL). The material has  $R_f$  values of 0.08 (EtOAc-THF, 1:1) and 0.16 (15% MeOH-CHCl<sub>3</sub>) on silica gel TLC. The UV spectrum gave  $\lambda_{\max}$  265 nm and  $\lambda_{\min}$  233 nm ( $\epsilon_{260}/\epsilon_{280}$  1.61) in absolute ethanol.

**Preparation of d[(MeO)<sub>2</sub>Tr]bzApCE.** A solution containing d[(MeO)<sub>2</sub>Tr]bzA (10.5 g, 16 mmol), methylphosphonic acid (32 mmol), and Dowex 50X pyridinium resin (0.5 g) in 80 mL of anhydrous pyridine was treated with dicyclohexylcarbodiimide (25 g, 121 mmol) for 3 days at 37 °C. Examination of the reaction mixture by LC showed essentially quantitative conversion of d[(MeO)<sub>2</sub>Tr]bzA to d[(MeO)<sub>2</sub>Tr]bzAp, which has a LC retention time of 22.8 min. The reaction mixture was treated with 80 mL of hydracrylonitrile for 2 days at 37 °C. After filtration and evaporation of the solvents, the residue was dissolved in 200 mL of ethyl acetate, and the solution was extracted with three (200-mL) portions of water. The ethyl acetate solution was dried over anhydrous sodium sulfate, concentrated to 50 mL, and chromatographed on a silica gel column (5.4 × 37 cm). The column was eluted with ether (1.5 L), ethyl acetate (1.5 L), and tetrahydrofuran (1.5 L). The resulting d[(MeO)<sub>2</sub>Tr]bzApCE weighed 5.4 g (6.84 mmol, 43%) after precipitation from tetrahydrofuran (100 mL) with hexane (500 mL). The material elutes from the LC column with 68% methanol-water and has silica gel TLC  $R_f$  values of 0.13 (EtOAc-THF, 1:1 v/v) and 0.27 (THF). The UV spectrum shows  $\lambda_{\max}$  279 and 234 nm and  $\lambda_{\min}$  258 and 223 nm ( $\epsilon_{234}/\epsilon_{279}$  1.44;  $\epsilon_{260}/\epsilon_{280}$  0.67) in 95% ethanol.

**Preparation of d[(MeO)<sub>2</sub>Tr]bzAp.** A solution containing d[(MeO)<sub>2</sub>Tr]bzApCE (3.79 g, 4.8 mmol) in 180 mL of dioxane and 36 mL of water was treated with 24 mL of 1 N

sodium hydroxide for 7 min. The solution was neutralized with Dowex 50X pyridinium resin and then was passed through a Dowex 50X pyridinium ion-exchange column (3 × 30 cm). The eluate was evaporated and the residue was rendered anhydrous by evaporation with pyridine. The resulting d[(MeO)<sub>2</sub>Tr]bzAp (2.9 g, 3.56 mmol) was obtained in 74% yield after precipitation from anhydrous ether. The material has  $R_f$  values of 0.00 (THF) and 0.36 (50% MeOH-CHCl<sub>3</sub>) on silica gel TLC. The UV spectrum showed  $\lambda_{\max}$  280 and 233 nm and  $\lambda_{\min}$  255 and 225 nm ( $\epsilon_{233}/\epsilon_{280}$  1.37;  $\epsilon_{260}/\epsilon_{280}$  0.67) in 95% ethanol.

The protecting groups were removed from a small sample of d[(MeO)<sub>2</sub>Tr]bzAp (80 mg, 0.1 mmol) by sequential treatment with concentrated ammonium hydroxide in pyridine and 80% acetic acid. The monomer dAp (1400  $A_{260}$  units, 0.09 mmol) was isolated by chromatography on a DEAE-Sephadex A-25 column (3 × 8.5 cm) using a linear gradient of ammonium bicarbonate (0.01–0.2 M, 600 mL). The monomer has the following  $R_f$  values on cellulose TLC: 0.45 (solvent A), 0.56 (solvent C), 0.67 (solvent F), and 0.44 (solvent I). The UV spectrum showed  $\lambda_{\max}$  259 nm and  $\lambda_{\min}$  227 nm ( $\epsilon_{260}/\epsilon_{280}$  6.13) in water, pH 7.0. The <sup>1</sup>H NMR spectrum was consistent with the structure of the monomer (Kan et al., unpublished experiments).

**Preparation of d(bz)ApCE.** A solution of d[(MeO)<sub>2</sub>Tr]bzApCE (1.58 g, 2 mmol) in 6.3 mL of methanol was treated with 25 mL of 80% acetic acid for 1.5 h. The solvents were evaporated, and the residue was repeatedly evaporated with toluene and tetrahydrofuran to remove acetic acid. The residue was precipitated from 20 mL of tetrahydrofuran by dropwise addition to 250 mL of hexane to give 0.95 g (1.95 mmol) of d(bz)ApCE in 98% yield. The material has  $R_f$  values of 0.09 (THF) and 0.25 (20% MeOH-CHCl<sub>3</sub>) on silica gel TLC and is eluted from the LC column with 12% methanol-water. The UV spectrum shows  $\lambda_{\max}$  280 and 233 nm (sh) and  $\lambda_{\min}$  247 nm ( $\epsilon_{233}/\epsilon_{280}$  0.65;  $\epsilon_{260}/\epsilon_{280}$  0.60) in 95% ethanol.

**Preparation of Dinucleoside Methylphosphonates.** The general procedure for the preparation of protected dinucleoside methylphosphonates is given in this section. Table I shows the specific reaction conditions and yields for each dimer. The protected nucleoside 3'-methylphosphonate and protected nucleoside or nucleoside 3'-methylphosphonate cyanoethyl ester were dried by evaporation with anhydrous pyridine. The condensing agent was added, and the reactants were taken up in anhydrous pyridine to give a 0.2 M solution. After completion of the reaction as indicated by TLC and/or LC, an equal volume of water was added and the solution was kept at room temperature for 30 min. The solvents were then evaporated and the residue was dissolved in ethyl acetate or chloroform. The organic solution was extracted with water and then dried over anhydrous sodium sulfate. After concentration, the organic solution was applied to a silica gel column (3 × 28 cm for a 1-mmol scale reaction). The column was

Table II: Chromatographic Mobilities and Ultraviolet Spectral Properties of Protected Dideoxyribonucleoside Methylphosphonates

dimer	mobility ( $R_f$ ) on silica gel TLC <sup>a</sup>			LC mobility <sup>c</sup> (%) methanol-water	UV spectral properties <sup>b</sup>	
	THF	10% MeOH-CHCl <sub>3</sub>	15% MeOH-CHCl <sub>3</sub>		$\lambda_{\max}$ (nm)	$\lambda_{\min}$ (nm)
d(MeOTr)TpTOAc	0.44	0.63, 0.53			267, sh 235	246
d(MeOTr)TpTpCE			0.28, 0.22		265, sh 235	245
d[(MeO) <sub>2</sub> Tr]bzApbzAOAc	0.34, 0.29		0.51, 0.43	68	281, sh 233	256
d[(MeO) <sub>2</sub> Tr]bzApbzApCE	0.09	0.32, 0.28	0.39, 0.35		281, sh 230	256
d(MeOTr)TpApbzAOAc	0.47	0.24, 0.19	0.59	62	276, 230, sh 260	247, 227
d[(MeO) <sub>2</sub> Tr]bzApTOAc	0.49, 0.41		0.52, 0.48	66	277, 235, sh 263	255, 227
						$\epsilon_{235}/\epsilon_{267} = 0.91$
						$\epsilon_{235}/\epsilon_{265} = 0.93$
						$\epsilon_{233}/\epsilon_{281} = 1.10$
						$\epsilon_{230}/\epsilon_{281} = 1.24$
						$\epsilon_{230}/\epsilon_{276} = 1.07$
						$\epsilon_{235}/\epsilon_{277} = 1.18$

<sup>a</sup> Two  $R_f$  values refer to the mobilities of the individual diastereoisomers. <sup>b</sup> Ultraviolet spectra were measured in 95% ethanol at room temperature. <sup>c</sup> Percentage of methanol in water required to elute the compound from the LC column (Du Pont Pharmaphase ODS).

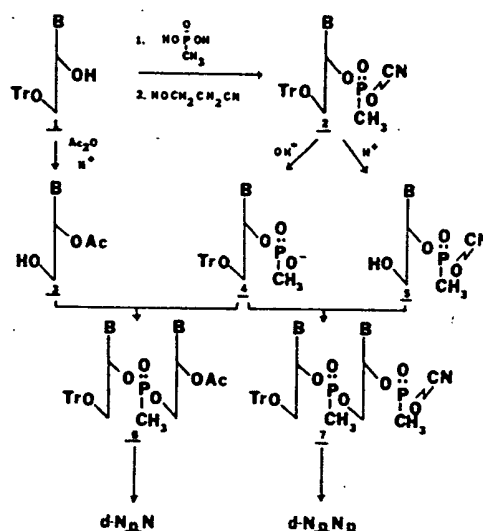
Table III: Chromatographic Mobilities and Ultraviolet Spectral Properties of Dideoxyribonucleoside Methylphosphonates

dimer	mobility on paper chromatography			UV spectral properties		
	$R_f$ (A)	$R_f$ (C)	$R_f$ (I)	$\lambda_{\max}$ (nm)	$\lambda_{\min}$ (nm)	$\epsilon_{260}/\epsilon_{280}$
dTpT		0.67	0.73	267	234	1.91
dApA		0.48	0.62	258	223	6.65
dApAp	0.30		0.38	258	227	4.52
dTpA	0.42	0.57	0.63	262	230	2.80
dApT	0.33	0.55	0.61	261	233	3.22

eluted with ethyl acetate, ethyl acetate-tetrahydrofuran (1:1 v/v), and tetrahydrofuran. The progress of the elution was monitored by silica gel TLC. Dimers terminating with a 3'-acetyl group separated into their individual diastereoisomers on the column and were eluted as pure isomer 1, a mixture of isomer 1 and 2, and pure isomer 2. The dimers were isolated as white solids, by precipitation from tetrahydrofuran solution upon addition of hexane. The  $R_f$  values on silica gel TLC, the mobilities on the LC column, and the ultraviolet spectral characteristics of the protected dimers are given in Table II.

The base-labile protecting groups were removed from the dimers by treatment with 50% concentrated ammonium hydroxide-pyridine solution for 3 days at 4 °C. Alternatively, the *N*-benzoyl protecting groups of dimers containing deoxyadenosine could be removed by treatment with 85% hydrazine hydrate in 20% acetic acid-pyridine buffer overnight at room temperature (Letsinger et al., 1968). This treatment also partially removed the 3'-*O*-acetyl group. The acetyl group was completely removed by further treatment with 50% concentrated ammonium hydroxide-pyridine solution for 2 h at 4 °C. After complete removal of solvents, the trityl protecting groups were removed by treatment with 80% acetic acid-methanol (8:2 v/v) solution at room temperature. The solvents were then removed and the dimers were chromatographed on Whatmann 3MM paper by using solvent A. The dimers were eluted from the paper with 50% aqueous ethanol. For dimers terminating with 3'-OH groups, the ethanol solutions were passed through small (0.5 × 1 cm) DEAE-cellulose columns to remove trace impurities eluted from the paper chromatogram. Dimers terminating with 3'-methylphosphonate groups were adsorbed to small DEAE-cellulose columns and then eluted with 0.5 M ammonium bicarbonate solution. The dimers were stored as standard solutions in 50% ethanol at 0 °C and were found to be completely stable under these conditions for at least 9 months. For physical and NMR studies, aliquots containing the required amount of dimer were evaporated to remove the ethanol and then lyophilized from water or D<sub>2</sub>O before use. The  $R_f$  values and UV spectral characteristics of the dimers are given in Table III. The <sup>1</sup>H NMR

Scheme I



spectra and tentative chemical shift assignments of the two diastereoisomers of dApA and dTpT are shown in Figure 1. Similar <sup>1</sup>H NMR spectra were obtained for dApT and dTpA (data not shown). The spectra are consistent with the structures of the dimers. The complete characterization of all these dimers by <sup>1</sup>H NMR spectroscopy will be described in a subsequent paper (Kan et al., unpublished experiments).

**Physical Studies and Interaction with Polynucleotides.** Ultraviolet and circular dichroism spectra were recorded respectively on a Cary 15 spectrophotometer and a Cary 60 spectropolarimeter with CD attachment. The continuous variation experiments, melting experiments, and circular dichroism experiments were carried out as previously described (Miller et al., 1971). The molar extinction coefficient of poly(U) is  $9.2 \times 10^3$  (265 nm) and of poly(dT) is  $8.52 \times 10^3$  (264 nm). The molar extinction coefficients of the dideoxyadenosine methylphosphonates were determined by comparing the absorption of a solution of the dimer at pH 7.4 with the absorption of the same solution at pH 1.0. The dimer extinction coefficient was then calculated from the observed hyperchromicity of the dimer at pH 1.0 by using an extinction coefficient for deoxyadenosine at pH 1.0 of  $14.1 \times 10^3$ .

## Results

**Preparation of Dinucleoside Methylphosphonates.** The synthetic route used to prepare the dinucleoside methylphosphonates is shown in Scheme I. 5'-(*p*-Methoxytrityl)thymidine and 5'-(*di-p*-methoxytrityl)-*N*-benzoyldeoxyadenosine were converted to the corresponding 3'-methylphosphonate  $\beta$ -cyanoethyl esters 2 by sequential reac-





Table IV: Hypochromicity of Dideoxyadenosine Methylphosphonate Analogues

compd	$\epsilon$ (M) <sup>a</sup>	% hypochromicity
dpA	$15.3 \times 10^3$	
dApA	$12.7 \times 10^3$	17
dApA <sup>1</sup>	$13.7 \times 10^3$	11.0
dApA <sup>2</sup>	$14.3 \times 10^3$	7.1
dApAp <sup>1</sup>	$13.0 \times 10^3$	13.3
dApAp <sup>2</sup>	$13.3 \times 10^3$	11.3

<sup>a</sup> The extinction coefficient per base residue at 258 nm was measured in 1 mM Tris-HCl, pH 7.4, at 27 °C.

was suppressed at low temperature. Alternatively, the *N*-benzoyl protecting groups of these dimers could be removed by treatment with hydrazine hydrate (Letsinger et al., 1968). The dimers were then purified by paper chromatography. The individual diastereoisomers of each deprotected dimer had the same chromatographic mobilities on paper chromatography in all solvent systems tested (see Table III).

**Ultraviolet and Hypochromicity Measurements.** The ultraviolet spectral properties of the dinucleoside methylphosphonates are recorded in Table III. Qualitatively, the spectra are similar to those of 3'-5'-linked dinucleoside monophosphates. The spectra of the individual diastereoisomers are qualitatively similar to each other.

Hypochromicity measurements for the dideoxyadenosine methylphosphonates were carried out in water at pH 7.4 and are shown in Table IV. The percent hypochromicity of the methylphosphonate dimers is from 4 to 10% lower than the percent hypochromicity of dApA. Each diastereoisomer has an unique molar extinction coefficient. The hypochromicity of isomer 1, the isomer eluted first from the silica gel column, is greater than that of isomer 2, reflecting differences in the extent of base-base overlap in these dimers.

**Circular Dichroism Spectra.** Differences in the extent and mode of base-stacking interactions are observed for individual diastereoisomers within a given dimer sequence as reflected by the CD spectra of the dimers. The profile of the CD spectrum of dApA<sup>1</sup> (Figure 2a) is qualitatively similar to that of the parent dinucleoside monophosphate, dApA (Miller et al., 1971). However, the magnitudes of the molecular ellipticity,  $[\theta]$ , at 267 and 270 nm of dApA<sup>1</sup> are approximately half of those found for dApA. A very dramatic difference in the CD spectrum of dApA<sup>2</sup> is observed. Only negative  $[\theta]$  is found at 250 nm and the amplitude of the molecular ellipticity is approximately threefold less than that of dApA<sup>1</sup>. Similar results were observed for dApAp<sup>1</sup> and dApAp<sup>2</sup> (data not shown).

In the case of dApT (Figure 2b), the profiles of the CD spectra of both isomers 1 and 2 are qualitatively similar to that of dApT (Cantor et al., 1970). However, the magnitudes of the ellipticity of the peak (272 nm) and trough (253 nm) of the dinucleoside methylphosphonate are less than those in the dinucleoside monophosphate. For dApT<sup>1</sup>, the peak is reduced 1.8-fold and the trough is reduced 1.3-fold compared to those of dApT while for dApT<sup>2</sup> the reductions are 8.1- and 5.0-fold.

The CD spectra of dTpA<sup>1</sup> and dTpA<sup>2</sup> (Figure 2c) show differences in both the magnitude of the molecular ellipticity and the position of the positive and negative bands. Isomer 2 has a CD spectrum which is virtually identical with that observed for dTpAp (Cantor et al., 1970). Isomer 1, on the other hand, has a lower magnitude of the  $[\theta]$  value, while the positions of the peak and trough are shifted to shorter wavelengths.

The CD results for dApT<sup>1</sup>, dApT<sup>2</sup>, dTpA<sup>1</sup>, and dTpA<sup>2</sup> are qualitatively similar to those obtained by Jensen & Reed

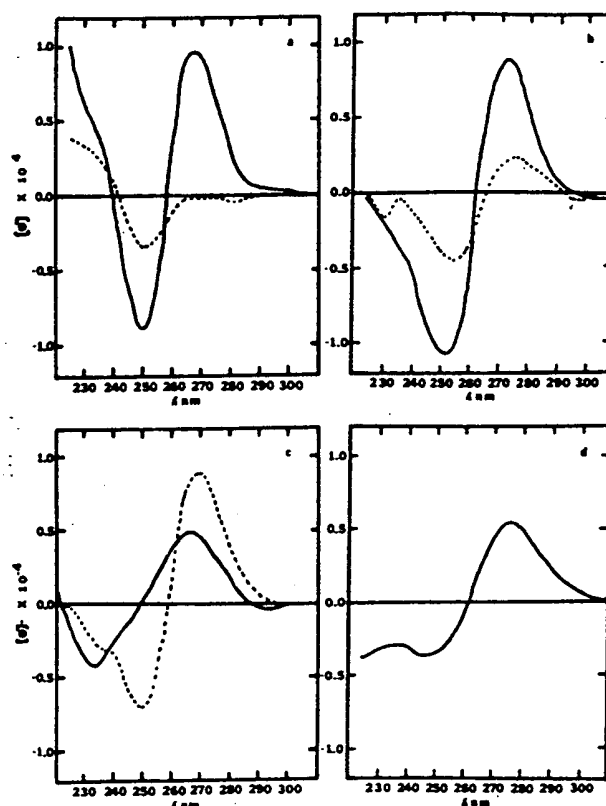


FIGURE 2: Circular dichroism spectra of (a) dApA<sup>1</sup> (—) and dApA<sup>2</sup> (---), (b) dApT<sup>1</sup> (—) and dApT<sup>2</sup> (---), (c) dTpA<sup>1</sup> (—) and dTpA<sup>2</sup> (---), and (d) dTpT in 10 mM Tris-HCl and 10 mM MgCl<sub>2</sub>, pH 7.5, at 27 °C. The molecular ellipticity,  $[\theta]$ , is given per base residue.

(1978) on the CD spectra of the separated diastereoisomers of the dinucleoside ethyl phosphotriesters, dAp(Et)T and dTp(Et)A. In the case of these triesters, one isomer has a spectrum which is almost identical with that of the corresponding dinucleoside monophosphate. The other isomer shows significant reductions in the magnitudes of both the positive and negative CD absorption bands. It is not possible at this time to make detailed comparisons between our results and those on the triesters, since the absolute configurations of the modified phosphate groups in the triesters are not known.

Figure 2d shows the CD spectrum of a 1:1 mixture of the diastereoisomers of dTpT. The spectrum of this mixture is clearly different from the spectrum of dTpT (Cantor et al., 1970). For dTpT, positive  $[\theta]$  occurs at 280 nm with a magnitude approximately 1.8-fold greater than that of the 275-nm band of dTpT. Similarly, dTpT shows negative  $[\theta]$  at 250 nm which is approximately 1.8-fold greater than that of the negative band at 245 nm in dTpT.

**Interaction of Dideoxyadenosine Methylphosphonates with Poly(U) and Poly(dT).** Both diastereoisomers of dApA form complexes with poly(U) at 0 °C. The mixing curves for dApA<sup>1</sup> and dApA<sup>2</sup> with poly(U) (Figure 3) show that complex formation occurs with a base stoichiometry of 2U:1A. Similar results were obtained for the interaction of dApAp<sup>1</sup> and dApAp<sup>2</sup> with poly(U) and for the interaction of dApA with poly(dT).

As shown in Figure 4, the methylphosphonate-poly-nucleotide complexes exhibit a cooperative thermal transition with a well-defined melting temperature. The melting temperature of the dApA<sup>1</sup>-poly(U) complex is 4.4 °C higher than that of the dApA<sup>2</sup>-poly(U) complex. A similar difference in melting temperatures for the dApAp-poly(U) complexes was

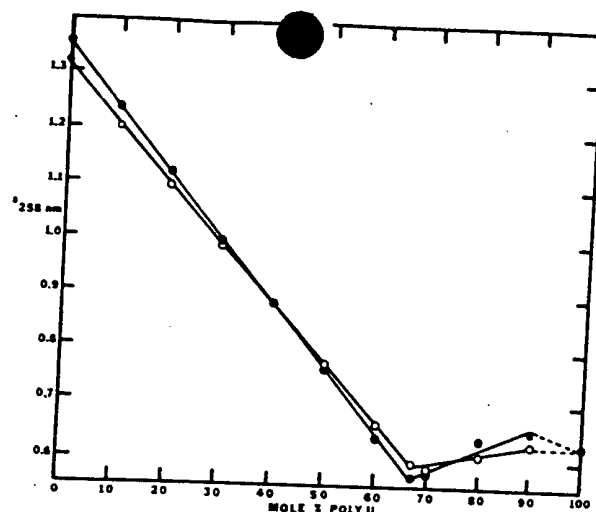


FIGURE 3: Mixing experiment between poly(uridylic acid) and dApA<sup>1</sup> (O) or dApA<sup>2</sup> (●) in 10 mM Tris and 10 mM MgCl<sub>2</sub>, pH 7.5, at 0 °C. The total nucleotide concentration is  $1 \times 10^{-4}$  M.

Table V: Melting Temperatures of Complexes Formed between Dideoxyadenosine Methylphosphonate Analogues and Poly(uridylic acid) or Poly(thymidylic acid)

complex <sup>a</sup>	$T_m$ (°C) [poly(U)] <sup>b</sup>	$T_m$ (°C) [poly(dT)]
dApA	7.0	9.2
dApA <sup>1</sup>	15.4	18.7
dApA <sup>2</sup>	19.8	18.4
dApAp <sup>1</sup>	13.5	
dApAp <sup>2</sup>	17.4	

<sup>a</sup> Complex stoichiometry: 2U:1A or 2T:1A. Total nucleotide concentration:  $5 \times 10^{-5}$  M. <sup>b</sup> 10 mM Tris-HCl and 10 mM MgCl<sub>2</sub>, pH 7.5.

also observed (Table V). Essentially no difference is observed between the  $T_m$  values of the two dApA-poly(dT) complexes, however.

Significant increases are observed in the thermal stabilities of the dinucleoside methylphosphonate-polynucleotide complexes as compared to similar complexes formed between dApA and poly(U) or poly(dT). The nonionic dApA forms complexes with  $T_m$  values 8.4 and 12.4 °C higher than that of dApA-poly(U), while the singly charged dApAp forms complexes with  $T_m$  values 6.5 and 10.4 °C higher than that of dApA-poly(U). Similarly, the complexes formed between dApA and poly(dT) each melt approximately 10 °C higher than the dApA-poly(dT) complex.

#### Discussion

Dinucleoside methylphosphonates are novel nucleic acid analogues in which the phosphodiester internucleoside linkage is replaced by a 3'-5'-linked internucleoside methylphosphonyl group. Unlike the dinucleoside methylenephosphonates prepared by Jones et al. (1970), the methylphosphonate analogues do not contain a negatively charged backbone and are nonionic molecules at pH 7. The methylphosphonate group is isosteric with respect to the phosphate group of dinucleoside monophosphates. Thus, these analogues should present minimal steric restrictions to interaction with complementary polynucleotides or single-stranded regions of nucleic acid molecules. Since the methylphosphonyl group is not found in naturally occurring nucleic acid molecules, this internucleoside linkage may be resistant to hydrolysis by various nuclease and esterase activities, and this has in fact been observed (Miller, unpublished data). These properties make analogues of this type potentially useful as vehicles for exploring the interactions of

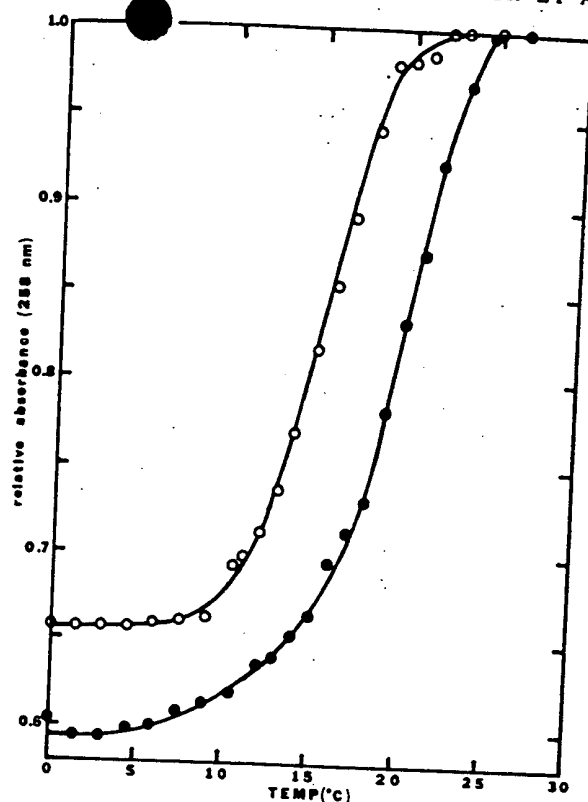


FIGURE 4: Melting curves of poly(U) plus dApA<sup>1</sup> (O) and poly(U) plus dApA<sup>2</sup> (●) in 10 mM Tris and 10 mM MgCl<sub>2</sub>, pH 7.5. The stoichiometry of each complex is 2U:1A and the total nucleotide concentration is  $5 \times 10^{-5}$  M.

selected oligonucleotide sequences with nucleic acids and nucleic acid related enzymes within the living cell (Miller et al., 1977).

The preparation of the oligonucleoside methylphosphonates follows the basic strategy used for the preparation of protected oligonucleotide phosphotriesters (Letsinger & Ogilvie, 1969). The synthetic scheme which has been adopted first involves preparation of a protected nucleoside 3'-methylphosphonate  $\beta$ -cyanoethyl ester (Scheme I). This two-step preparation can be carried out in a one-flask reaction and proceeds in high overall yield. Since the product is readily purified by silica gel column chromatography, multigram quantities of this key intermediate can be prepared. By selective removal of the 5'-trityl group or the  $\beta$ -cyanoethyl group, chain extension can proceed in either direction. Thus, compound 1 in Scheme I serves as a basic building block for the preparation of longer oligomers. This type of synthetic scheme was originally developed by Catlin & Cramer (1973) for the preparation of oligonucleotide  $\beta,\beta,\beta$ -trichloroethyl phosphotriesters and has more recently been used by Narang and co-workers (Itakura et al., 1975; Stawinski et al., 1977) for the preparation of oligonucleotide *p*-chlorophenyl phosphotriesters. This procedure also allows the preparation of specifically <sup>13</sup>C-enriched dimers by use of [<sup>13</sup>C]methylphosphonic acid in the synthesis of 1. Dimers and oligomers containing [<sup>13</sup>C]methylphosphonate groups could be very useful for probing the physical and biological properties of oligonucleoside methylphosphonates by nuclear magnetic resonance spectroscopic techniques (Cheng et al., unpublished experiments).

In the present study (Scheme I), the  $\beta$ -cyanoethyl group was removed from 1 and chain extension was continued in the 3' direction. Two types of condensation reactions were carried out: (1) condensation with a 3'-O-acetylated nucleoside to give dimers with the general structure 6 and (2) condensation with

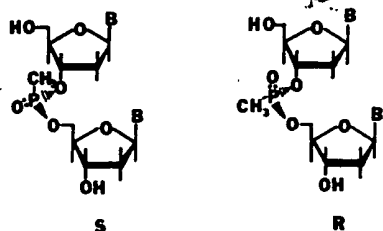


FIGURE 5: Diastereoisomers of dideoxyribonucleoside methylphosphonates.

a nucleoside 3'-methylphosphonate  $\beta$ -cyanoethyl ester to give dimers with general structure 7. The latter type of dimer can be further extended by removal of the  $\beta$ -cyanoethyl group, followed by condensation with other oligonucleoside methylphosphonate blocks. In this way, oligonucleoside methylphosphonates containing up to four deoxyadenosine residues and up to nine thymidine residues have been prepared (P. S. Miller and J. Yano, unpublished results).

Different condensing agents were used in these reactions, including dicyclohexylcarbodiimide (DCC), triisopropylbenzenesulfonyl chloride (TPSCI), and mesitylenesulfonyl tetrazolide (MST). The order of condensing efficiency was found to be MST > TPSCI > DCC. Although DCC did bring about condensation, several days at elevated temperatures were required and the yields were quite low. Considerable improvement in reaction yield was obtained when TPSCI was used. However, again prolonged reaction periods were required and noticeable buildup of side products was observed. The reagent of choice for these reactions is MST. The reaction occurs within a period of several hours, with little or no side products. The efficiency of a particular condensing agent depends not only upon its structure but also upon the nature of the phosphorous-containing substituent which is activated. Thus, when MST was used as a condensing agent, we observed that reactions involving nucleoside 3'-methylphosphonates or nucleoside 3'-ethyl phosphates usually proceed in lower yield than those involving nucleoside 3'-*p*-chlorophenyl phosphates.

The ability to separate the individual diastereoisomers of each dimer sequence allowed examination of the effect of the configuration of the phosphonyl methyl group on the overall dimer conformation. As shown in Figure 5, the isomers differ in configuration at the internucleoside linkage with the methyl group, assuming either a pseudoaxial (*S*) or pseudoequatorial (*R*) position when the dimers are drawn in a stacked conformation. The unique conformational properties of each diastereoisomer of dApA and dApAp are most readily seen by examining the percent hypochromicity of each diastereoisomer (Table IV). Isomer 1 of both dApA and dApAp exhibits a greater percent hypochromicity than does isomer 2 of this series. Since the percent hypochromicity is related to the extent of base-base overlap in dimers of this type (Ts'o, 1974), the result suggests that dApA<sup>1</sup> and dApAp<sup>1</sup> are more highly stacked in solution than are dApA<sup>2</sup> and dApAp<sup>2</sup>. Comparison of the percent hypochromicities of the methylphosphonate dimers with that of dApA shows that these dimers are less stacked than the parent dinucleoside monophosphate. A similar result was observed for the methyl and ethyl phosphotriesters of dApA (Miller et al., 1971). Thus, nonionic methyl or ethyl phosphotriester or methylphosphonate internucleoside linkages appear to perturb the stacking interactions between the bases in these dimers.

The circular dichroism spectra of dinucleoside monophosphates are indicators of both the extent and mode of base stacking, as well as the population of right-handed vs. left-handed stacks (Kondo et al., 1972; Ts'o, 1974). The CD

spectra of each diastereoisomer for the methylphosphonate dimer sequences dApA, dApAp, dApT, and dTpA suggest that each diastereoisomer has a unique stacking mode in solution. The profiles of the CD spectra of dApA<sup>1</sup> and dApAp<sup>1</sup> are very similar to those of dApA and rApA and differ only in the magnitude of the molecular ellipticity. This result and the results of the hypochromicity measurements suggest that the stacking modes of the bases in these dimers are similar to those of dApA and rApA. On the other hand, the profiles of the CD spectra of dApA<sup>2</sup> and dApAp<sup>2</sup> are quite different. The magnitudes of the molecular ellipticities of dApA<sup>2</sup> and dApAp<sup>2</sup> are greatly diminished, with complete loss of  $[\theta]$  at 270 nm. Since the hypochromicity measurements suggest that the bases in these dimers have substantial overlap, the mode of stacking in these dimers must be quite different from that found for isomer 1 or for dApA. The magnitude of the molecular ellipticity in dimers of this type is sensitive to the angle,  $\theta$ , between the transition dipoles of the bases (Ts'o, 1974). The value of the molecular ellipticity is greatest when  $\theta$  is 45° and diminishes to 0 when  $\theta$  is 0, 90, or 180°. Thus, the most reasonable interpretation of the CD results is that, in dApA<sup>1</sup> and dApAp<sup>1</sup>, the bases tend to orient in an oblique manner, while, in dApA<sup>2</sup> and dApAp<sup>2</sup>, the bases tend to orient in a parallel or perpendicular manner. This interpretation is supported by the base-base stacking patterns as determined by <sup>1</sup>H NMR spectroscopy (Kan et al., unpublished experiments). The substantial change in the CD profile of dApA<sup>2</sup> rather than a simple diminution of the amplitude of the  $[\theta]$  values suggests that variation of the population of right-handed vs. left-handed stacks would not provide an adequate explanation of the CD results.

The CD spectra of dApT isomers 1 and 2 have the same shape as the CD spectrum of dApT but with diminished molecular ellipticity. For dTpA, the spectrum of isomer 2 is identical with that of dTpAp, while the spectrum of isomer 1 shows diminished  $[\theta]$  values of the peak and trough regions. Thus, the stacking modes in these methylphosphonate dimers are expected to be basically similar to the stacking modes of the parent dinucleoside monophosphates but with perhaps different degrees of base-base overlap or different populations of right- and left-handed stacks.

The dimer dApA forms stable complexes with both polyribo- and polydeoxyribonucleotides. These poly(U) and poly(dT) complexes have greater stability than similar complexes formed by the parent dinucleoside monophosphate, dApA. Similar observations have previously been made for triple helix formation between the alkyl phosphotriesters dAp(Me)A or dAp(Et)A and poly(U) (Miller et al., 1971), for duplex formation between oligonucleotide triesters and tRNA (Miller et al., 1974), and for helical duplex formation between the octa-thymidylate ethyl phosphotriester d[Tp(Et)]<sub>7</sub>T and poly(dA) (Pless & Ts'o, 1977). It should be noted, however, that d[Tp(Et)]<sub>7</sub>T, in contrast to dApA, exhibits selective binding to polydeoxyribonucleotides vs. polyribonucleotides in duplex formation.

Our previous analyses indicate that the increased stability of the complexes formed between nonionic oligomers and complementary polynucleotides results from the reduction in charge repulsion between the nonionic backbone of the oligomer and the negatively charged sugar-phosphate backbone of the polynucleotide (Miller et al., 1971; Pless & Ts'o, 1977). Although both dApAp and dApA possess a formal negative charge, the dApAp-poly(U) complexes are more stable than the dApA-poly(U) complex. The 3'-terminal methylphosphonate group of dApAp is free to rotate away from the

negatively charged phosphate backbone of poly(U) without disrupting the base-pairing and base-stacking interactions in the complex. In contrast, repulsion between the negative charge of the phosphodiester linkage in dApA and the polymer backbone directly opposes base pairing and stacking. Thus, the presence of a negative charge at the internucleotide linkage contributes much more effectively to the charge repulsion effect between the dimers and polynucleotides.

Under the conditions of the present experiments, the  $T_m$  values of dAp(Me)A-poly(U) and dAp(Et)A-poly(U) are 13 and 12 °C, respectively. These  $T_m$  values are lower than those of dApA and dApAp complexes with poly(U). These results suggest that the increasing size of the methyl and ethyl side chains in the phosphotriester dimers may provide a greater steric hindrance to complex formation. The methyl group of the phosphonate dimers should be only slightly larger in size than the oxygen of the phosphate group and thus would be expected to have the least steric effect. A similar phenomenon has been observed when the stabilities of poly(U) complexes with the ethyl phosphotriester and methylphosphonate analogues of dApApA are compared (Jayaraman et al., 1979).

The differences in the conformations of the individual diastereoisomers of dApA and dApAp are reflected in their interactions with poly(U). For each dimer, the diastereoisomer with greater base-base overlap (isomer 1) forms a complex of lower stability with poly(U). In a previous analysis of the influence of C-2' substituents of adenine polynucleotides on the  $T_m$  values of the helices (Alderfer et al., 1974), we reasoned that the conformation free-energy difference ( $F_D - F_S$ ) at the melting temperature is directly related to the  $T_m$  value, where  $F_D$  represents the free energy of the double-stranded duplex and  $F_S$  represents the free energy of the base-stacked single strand. The values of  $F_D - F_S$  reflect the conformation of the duplex state and the single-stranded state. The data indicate that  $F_D - F_S$  for isomer 1 of dApA or dApAp is slightly less than  $F_D - F_S$  for isomer 2 of dApA or dApAp. This reduction may reflect a higher  $F_S$  value of isomer 1 since this isomer indeed has a greater degree of stacking, assuming that  $F_D$  for isomer 1 and isomer 2 remains the same. In contrast to the behavior with poly(U), both diastereoisomers of dApA form complexes with poly(dT) which have similar  $T_m$  values. Since the geometry of the triple helix of dApA-2-poly(U) is likely to be different than the geometry of the dApA-2-poly(dT) triple helix, the difference in  $F_S$  of isomer 1 vs.  $F_S$  of isomer 2 may be compensated by a difference in  $F_D$  of isomer 1 vs.  $F_D$  of isomer 2.

The studies reported in this paper have shown that dideoxyribonucleotide analogues containing nonionic 3'-5' internucleoside methylphosphonate linkages can be readily synthesized. The configuration of the methyl group in the backbone of these dimers influences their conformation in solution and their ability to form complexes with complementary polynucleotides. More detailed descriptions of the conformations of these molecules based on NMR spectroscopic results will be presented in subsequent papers in this series (Kan et al., unpublished experiments; Cheng et al., unpublished experiments). In addition, preliminary studies have shown that oligodeoxyribonucleoside methylphosphonates are resistant to nuclease hydrolysis, are taken up in intact form by mammalian cells in culture, and can exert specific inhibitory effects on cellular DNA and protein synthesis. Unlike 2'-O-methyl oligonucleotide ethyl phosphotriesters, the methylphosphonates appear to have relatively long half-lives within the cells. Thus, oligonucleoside methylphosphonates of specific sequence could complement oligonucleotide phosphotriesters as probes and

regulators of nucleic acid function within living cells.

#### Added in Proof

Recently Agarwal & Riftina (1979) reported the syntheses of some dideoxyribonucleoside methylphosphonates using synthetic procedures different from those reported here.

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## Inhibition of Intractable Nucleases with Ribonucleoside-Vanadyl Complexes: Isolation of Messenger Ribonucleic Acid from Resting Lymphocytes<sup>†</sup>

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**ABSTRACT:** Human lymphocyte lysates prepared by detergent treatment of intact, normal resting cells contain ribonucleases that are insensitive to many inhibitors commonly used with eucaryotic cells. Phenol-extracted ribonucleic acid (RNA) obtained directly from unfractionated cytoplasm is sometimes degraded, but after fractionation of the cytoplasmic material by sucrose density gradient centrifugation, the polyadenylated RNA, in particular, is inevitably destroyed. An extensive survey of ribonuclease inhibitors, undertaken as a consequence, indicated that the complexes formed between the oxovanadium ion and the four ribonucleosides were unique in their ability to suppress lymphocyte nuclease activity. It proved possible to isolate intact ribosomal RNA and polyadenylated messenger RNA from lymphocyte cytoplasm fractionated on sucrose

gradients when 2.5 mM each of the four ribonucleoside-vanadyl complexes was used throughout the procedure. The data showed that the size distribution of poly(A)-bearing RNA remained unchanged, with a peak at ~16 S under denaturing conditions, regardless of whether the mRNA was originally associated with polysomes or was nonpolysome bound. The cytoplasmic RNAs were completely free of contamination by either intact nuclear RNA or nuclear fragments. Furthermore, exogenous globin mRNA mixed with lymphocytes and reisolated together with endogenous cytoplasmic polyadenylated RNA was fully translatable only when ribonucleoside-vanadyl complexes were employed during the preparation. The use of this inhibitor should therefore be considered for all tissues in which ribonucleases impede isolation of intact RNA.

The isolation of intact RNA from most animal cells relies on the use of exogenous ribonuclease inhibitors. Many substances including diethyl pyrocarbonate, polyvinyl sulfate, heparin, bentonite, macaloid, an assortment of ribonucleotides, sodium dodecyl sulfate, and proteinase K have been employed routinely for this purpose (Poulson, 1977). Recent investigations, however, have revealed that pancreatic ribonuclease (Jones, 1976) as well as other nucleases in crude cell homogenates (Gray, 1974; Egberts et al., 1977) retains enzymatic activity in the presence of these agents. Although the studies were carried out under test conditions removed from those actually confronted in preparative situations, in all cases the warning implicit in these findings is unmistakable. The ability to obtain intact RNA from HeLa cells (Milcarek et al., 1974), hen oviducts (Schimke et al., 1974), or fibroblasts (Johnson et al., 1974), for example, is probably a fortuitous event stemming from low endogenous levels of nucleases that are sensitive to one or more of the substances listed above. It is also possible that subcellular compartmentalization maintains the separation between RNA and RNases more effectively during lysis of some cells than during disruption of others. Clearly, the ability to carry out quantitative studies of RNA, particularly under conditions in which protein denaturation and deproteinization are incompatible with the aim of the experiment, depends upon a restricted choice of biological material. Those systems in which the nucleases are known to be intractable (Spradling et al., 1975; Cooper & Kay, 1969) receive little attention.

In order to work with resting lymphocytes, it was necessary to overcome this problem. Toward this end, we have tested a number of ribonuclease inhibitors for their ability to protect RNA in crude lysates of these cells. The criteria for choosing suitable inhibitors were the following: (1) the substance must be compatible with cell lysis techniques in which subcellular organelles are to be purified; (2) RNA must not leak out of the nuclei; (3) cytoplasmic components such as ribonucleo-protein particles and polysomes should be unaffected; and (4) RNA must remain undegraded in disrupted cells during sucrose gradient centrifugation of cytoplasm, a technique requiring several hours in the presence of proteins. Of the inhibitors tested, only one related set, the complexes formed between  $\text{VO}_2^{+}$  and each of the four ribonucleosides, proved satisfactory. The use of the mixed vanadyl complexes not only facilitated the isolation of structurally intact, translatable RNA but also increased the yield of polyadenylated mRNA from resting lymphocytes fourfold.

### Materials and Methods

**Materials.** Iodoacetic acid, EGTA,<sup>1</sup> diethyl pyrocarbonate, and the ribonucleoside 2',3'-monophosphates (cyclic) of adenine, guanine, cytosine, and uracil were purchased from Sigma. Other compounds and their suppliers were as follows: polyuridylic acid, Schwarz/Mann; vanadyl sulfate, Fisher; proteinase K, E. Merck Darmstadt; ribonucleoside 3',5'-bis-

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<sup>1</sup> Abbreviations used: EGTA, ethylene glycol bis( $\beta$ -aminoethyl ether)- $N,N'$ -tetraacetic acid; Hepes,  $N$ -2-hydroxyethylpiperazine- $N'$ -2-ethanesulfonic acid.



# Biochemical and Biological Effects of Nonionic Nucleic Acid Methylphosphonates†

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**ABSTRACT:** Oligodeoxyribonucleoside methylphosphonates with base sequences complementary to the anticodon loop of tRNA<sup>Lys</sup> and to the -ACCA-OH amino acid accepting stem of tRNA were prepared by chemical synthesis. Oligodeoxyadenosine methylphosphonates form stable, triple-stranded complexes with both poly(U) and poly(dT). These analogues selectively inhibit cell-free aminoacylation of tRNA<sup>Lys</sup><sub>E.coli</sub> but have no effect on aminoacylation of tRNA<sup>Lys</sup><sub>rabbit</sub>. The extent of inhibition is temperature dependent and parallels the ability of the oligomer to bind to poly(U), which suggests that inhibition occurs as a result of oligomer binding to the -UUU-anticodon loop of tRNA<sup>Lys</sup><sub>E.coli</sub>. The failure of the oligodeoxyadenosine methylphosphonates to inhibit tRNA<sup>Lys</sup><sub>rabbit</sub> aminoacylation suggests that there may be a difference between the

structure of tRNA<sup>Lys</sup> or its interaction with aminoacyl synthetase in the *Escherichia coli* and rabbit systems. The oligodeoxyadenosine analogues also effectively inhibit polyphenylalanine synthesis in cell-free translation systems derived from both *E. coli* and rabbit reticulocytes. The extent of inhibition parallels the  $T_m$  values of the oligo(A) phosphonate-poly(U) complexes and suggests that the inhibition is a consequence of complex formation with the poly(U) message. Tritium-labeled oligodeoxyribonucleoside methylphosphonates with a chain length of up to nine nucleotidyl units are taken up intact by mammalian cells in culture. All the oligomer analogues tested inhibited, to various extents, colony formation by bacterial, hamster, and human tumor cells in culture.

Nonionic oligonucleotide analogues have been shown to be useful nucleic acid analogues for probing nucleic acid sequence-function relationships both in biochemical experiments and in living cells. Previous reports from this laboratory have described the interaction of nonionic, oligonucleotide ethyl phosphotriesters with transfer RNA (Miller et al., 1974) and the effects of these analogues on cell-free aminoacylation of tRNA (Barrett et al., 1974). A trinucleotide analogue, G<sup>m</sup>(Et)G<sup>m</sup>(Et)U<sup>1</sup> was shown to be taken up by mammalian cells in culture and to have specific inhibitory effects on cellular protein synthesis and cell growth (Miller et al., 1977).

Recently we described the syntheses of a series of novel nonionic oligonucleotide analogues, the dideoxyribonucleoside methylphosphonates (Miller et al., 1979). These analogues have an isosteric, 3'-5'-linked methylphosphonate group which replaces the normal phosphodiester linkage of nucleic acids. Extensive physical studies by ultraviolet, circular dichroism, and nuclear magnetic resonance spectroscopic techniques revealed that the conformation of these analogues is similar to those of the corresponding phosphodiester and that the analogues form stable complexes with complementary polynucleotides (Miller et al., 1979; Kan et al., 1980). Since these phosphonate analogues can penetrate mammalian cells and the methylphosphonate linkage is resistant to cleavage by a variety of nucleases, it is of interest to determine if these analogues could be used as probes of the sequence-function relation of nucleic acids in living cells. In this paper we report the synthesis of a series of oligonucleoside methylphosphonates whose base sequences are complementary to the anticodon loops of tRNA<sup>Lys</sup> species and to the -ACCA-OH amino acid accepting stem of tRNA. The effects of these analogues on cell-free aminoacylation and cell-free protein synthesis were studied. The uptake of selected analogues by mammalian cells in culture and the effects of these compounds on bacterial and

mammalian cell growth are reported.

## Experimental Section

**Materials.** Nucleosides were purchased from P-L Biochemicals and were checked for purity by paper chromatography before use. *N*-Benzoyldeoxyadenosine, *N*-isobutyryldeoxyguanosine, their 5'-*O*-dimethoxytrityl derivatives, and 5'-*O*-(methoxytrityl)thymidine were prepared according to published procedures (Schaller et al., 1963; Büchi & Khorana, 1972). d-[(MeO)<sub>2</sub>Tr]bzApbzApCNEt, d-[(MeO)<sub>2</sub>Tr]bzApbzAOAc, d-[(MeO)Tr]TpTpCNEt, d-ApT, d-Ap[<sup>3</sup>H]T, d-TpT, and d-Tp[<sup>3</sup>H]T were synthesized by procedures previously described (Miller et al., 1979). Dimethyl methylphosphonate (K & K Laboratories) and benzenesulfonic acid (Eastman) were used without further purification. Hydroacrylonitrile (Eastman) was dried over 4-Å molecular sieves. Methylphosphonic acid dipyrindinium salt and mesitylenesulfonyl tetrazolidine were prepared as previously described (Miller et al., 1979). Anhydrous pyridine was prepared by refluxing reagent-grade pyridine (3 L) with chlorosulfonic acid (40 mL) for 7 h, followed by distillation onto sodium hydroxide pellets (40 g). After being refluxed for 7 h, the pyridine was distilled onto 4-Å molecular sieves and stored in the dark.

Silica gel column chromatography was carried out by using Baker 3405 silica gel (60-200 mesh). Thin-layer silica gel chromatography (TLC) was performed on E. Merck silica gel 60 F<sub>254</sub> plastic-backed TLC sheets (0.2 mm thick). High-pressure liquid chromatography (LC) was carried out by using a Laboratory Data Control instrument on columns (2.1 mm × 1 m) packed with HC Pello-sil (Whatman, Inc.). The columns were eluted with a linear gradient (40 mL total) of chloroform to 20% (v/v) methanol in chloroform at a flow rate

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<sup>1</sup> Abbreviations used: Np(Et)N, an oligonucleotide ethyl phosphotriester; d-NpNpN, oligodeoxyribonucleotide analogues containing 3'-5' internucleoside methylphosphonate linkages (in this abbreviation an italic p represents the methylphosphonate linkage); MST, mesitylenesulfonyl tetrazolidine. The symbols used to represent protected nucleosides and oligonucleoside methylphosphonates follow the IUPAC-IUB Commission on Biochemical Nomenclature (1970) recommendations.

of 1 mL/min. Ultraviolet spectra were recorded on a Cary 14 or a Varian 219 ultraviolet spectrophotometer with a temperature-controlled cell compartment. The following extinction coefficients (260 nm) were used: dT, 9100; d-[(MeO)Tr]T, 10 200; d-[(MeO)<sub>2</sub>Tr]bzA, 12 500; d-bzA, 10 600; d-[(MeO)<sub>2</sub>Tr]ibuG, 17 400; d-ibuG, 16 700. Paper chromatography was carried out on Whatman 3 MM paper using solvent A: 2-propanol-concentrated ammonium hydroxide-water (7:1:2 v/v).

**Preparation of d-[(MeO)<sub>2</sub>Tr]ibuGpCNEt.** d-[(MeO)<sub>2</sub>Tr]ibuG (12 g, 18.7 mmol) and the pyridinium salt of methylphosphonic acid (21 mmol) were dried by evaporation with anhydrous pyridine (4 × 20 mL) and the residue in 40 mL of pyridine was treated with 2,4,6-triisopropylbenzenesulfonyl chloride (12.7 g, 42 mmol) for 8 h at room temperature. Hydracrylonitrile (4.5 g, 63 mmol) and 2,4,6-triisopropylbenzenesulfonyl chloride (0.61 g, 2 mmol) were added and the reaction mixture was kept at room temperature. After 2 days the reaction mixture was poured into 500 mL of ice-cold 5% NaHCO<sub>3</sub> solution. The solution was extracted with ethyl acetate (2 × 250 mL) and the combined extracts were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. Examination of the extract by TLC showed the presence of both d-[(MeO)<sub>2</sub>Tr]ibuGpCNEt (*R<sub>f</sub>* 0.31, silica gel TLC, 10% MeOH-CHCl<sub>3</sub>) and d-ibuGpCNEt (*R<sub>f</sub>* 0.14, silica gel TLC, 10% MeOH-CHCl<sub>3</sub>). After concentration the ethyl acetate extract was chromatographed on silica gel (4 × 35 cm) by using ether (1 L) and a 0-20% linear gradient of methanol in chloroform (1.6 L total) as solvents. d-[(MeO)<sub>2</sub>Tr]ibuGpCNEt (2.75 mmol) was obtained in 15% yield while d-ibuGpCNEt (2.46 mmol) was obtained in 13% yield. Additional d-[(MeO)<sub>2</sub>Tr]ibuGp (3.69 mmol, 20%) was obtained from the aqueous bicarbonate solution after extraction with chloroform (2 × 200 mL).

**Preparation of Protected Oligonucleoside Methylphosphonates.** The same general procedures were used as previously described for the preparation of dinucleoside methylphosphonates (Miller et al., 1979). The specific conditions used in the condensation reactions and the yields obtained after silica gel column chromatography are given in Table I. The ultraviolet spectroscopic characteristics and the mobilities of the protected oligonucleotides on silica gel TLC and silica gel high-pressure LC are given in Table II.

**Preparation of Oligonucleoside Methylphosphonates.** The protecting groups were removed from the blocked oligonucleoside methylphosphonates by using conditions described previously (Miller et al., 1979). In the case of the dA-containing oligomers, the *N*-benzoyl groups were removed by treatment with hydrazine (Miller et al., 1979). The oligomers were purified by preparative paper chromatography using solvent A. For the <sup>3</sup>H-labeled oligothymidine methylphosphonates, d-(Tp)<sub>n</sub>[<sup>3</sup>H]T, the condensation reactions containing d-[(MeO)Tr](Tp)<sub>n</sub> plus [<sup>3</sup>H]TOAc were run on 0.01 (*n* = 1) and 0.005 (*n* = 4 and 8) mmol scales while d-GpGp[<sup>3</sup>H]T was prepared on a 0.012-mmol scale. The protecting groups were removed without isolation of the protected <sup>3</sup>H-labeled oligomers and the entire reaction mixture was chromatographed on paper. The oligonucleoside methylphosphonates were eluted from the paper with 50% aqueous ethanol. The ethanol solutions were passed through DEAE-cellulose columns (0.5 × 1 cm) and stored at 0 °C. The following overall yields were obtained: d-(Tp)<sub>n</sub>[<sup>3</sup>H]T (*n* = 1, 41%; *n* = 4, 22%; *n* = 8, 17%) and d-GpGp[<sup>3</sup>H]T (15%). The UV spectral properties and chromatographic mobilities of the oligonucleoside methylphosphonates are given in Table III. For use in the physical, biochemical, and biological ex-

periments described below, aliquots containing the required amount of oligomer were evaporated to dryness, and the oligomer was dissolved in the buffer used in the particular experiment.

**Interaction of Oligodeoxyadenylate Methylphosphonates with Polynucleotides.** The continuous variation experiments and melting experiments were carried out as previously described (Miller et al., 1971). The extinction coefficients of the oligomers were determined by comparing the absorption of a solution of the oligomer in water at pH 7.0 to the absorption of the same solution at pH 1.0. The oligomer extinction coefficient was calculated from the observed hyperchromicity of the oligomer at pH 1.0 by using the following extinction coefficients: dA, pH 1.0, 14.1 × 10<sup>3</sup>; dG, pH 1.0, 12.3 × 10<sup>3</sup>. The molar extinction coefficient of poly(U) is 9.2 × 10<sup>3</sup> (265 nm) and of poly(dT) is 8.52 × 10<sup>3</sup> (264 nm).

**Cell-Free Aminoacylation.** (1) *E. coli* System. Unfractionated *Escherichia coli* tRNA was purchased from Schwarz/Mann and unfractionated *E. coli* aminoacyl synthetase was purchased from Miles Laboratories, Inc. Reactions were run in 60 μL of buffer containing 100 mM Tris-HCl, pH 7.4, 10 mM Mg(OAc)<sub>2</sub>, 5 mM KCl, 2 mM ATP, 4 μM <sup>3</sup>H-labeled amino acid, 1.8 μM tRNA<sub>*E. coli*</sub>, and 0-100 μM oligonucleotide, following the procedure of Barrett et al. (1974). Reactions were initiated by addition of 4 μg of aminoacyl synthetase. Aliquots (10 μL) were removed at various times and added to 1 mL cold 10% trichloroacetic acid and the resulting precipitate was filtered on Whatman G/F filters. After being washed with four (1 mL) portions of 2 N HCl and four (1 mL) portions of 95% ETOH, the filters were dried and counted in 7 mL of New England Nuclear 949 scintillation mixture.

(2) *Rabbit Reticulocyte System.* A rabbit reticulocyte cell-free translation system was purchased from New England Nuclear (lot no. J1157AW). Reactions were run in 12.5 μL of buffer containing 1 μL of the translation mixture, 79 mM potassium acetate, 0.6 mM magnesium acetate, 57 μM [<sup>3</sup>H]lysine, and 50 μM oligomer. The reactions were initiated by addition of 5 μL of reticulocyte lysate and were assayed as described for the *E. coli* system.

**Cell-Free Protein Synthesis.** (1) *E. coli* System. A cell-free protein synthesizing system was isolated from *E. coli* B cells (S-30) according to the procedure of Nirenberg (1963). The system incorporates 300 pmol of [<sup>3</sup>H]phenylalanine/mg of S-30 protein after 15-min incubation at 37 °C when poly(U) is used as a message.

(2) *Rabbit Reticulocyte.* The reticulocyte translation system prepared by New England Nuclear was used. For the translation of globin mRNA, the reactions were run in 12.5 μL of buffer containing 1 μL of the translation mixture, 0.10 μg of globin mRNA (Miles Laboratories), 79 mM potassium acetate, 0.2 mM magnesium acetate, 0-50 μM oligomer, and 20.5 μM [<sup>3</sup>H]leucine. For the translation of poly(U), the reactions were run in 12.5 μL of buffer containing 1 μL of the translation mixture, 120 mM potassium acetate, 0.8 mM magnesium acetate, 367 μM poly(U), 0-200 μM oligomer (base concentration), and 32 μM [<sup>3</sup>H]phenylalanine. Reactions were initiated by addition of 5 μL of reticulocyte lysate. Aliquots (2 μL) were removed at various times and added to 1.0 mL of bovine serum albumin (100 μg) solution. The protein was precipitated by heating with 1 mL of 10% trichloroacetic acid at 70 °C, filtered on G/F filters, and counted in 7 mL of Betafluor.

**Uptake of Oligodeoxyribonucleoside Methylphosphonates.** The uptake of d-Ad[<sup>3</sup>H]T, d-GpGp[<sup>3</sup>H]T, and d-(Tp)<sub>n</sub>[<sup>3</sup>H]T



Table I: Preparation of Protected Oligodeoxyribonucleoside Methylphosphonates

3'-methylphosphonate com (mmol)	5'-OH component (mmol)	MST (mmol)	product (mmol)	yield (%)
d-[(MeO) <sub>2</sub> Tr]ibuGp (0.50)	d-ibuGpCNEt (0.50)	2.0	d-[(MeO) <sub>2</sub> Tr]ibuGpibuGpCNEt (0.82)	16
d-[(MeO) <sub>2</sub> Tr]ibuGp (1.0)	d-bzAOAc (1.5)	4.0	d-[(MeO) <sub>2</sub> Tr]ibuGpzbAOAc (0.42)	42
d-[(MeO)Tr]TpTp (0.33)	d-TpTpCNEt (0.50)	1.6	d-[(MeO)Tr]TpTpTpTpCNEt (0.168)	50
d-[(MeO)Tr]Tp(Tp) <sub>2</sub> TPCNEt (0.0324)	d-Tp(Tp) <sub>2</sub> TPCNEt (0.0524)	0.16	d-[(MeO)Tr]Tp(Tp) <sub>2</sub> TPCNEt (0.0138)	43
d-[(MeO) <sub>2</sub> Tr]ibuGpibuGp (0.07)	d-TOAc (0.15)	0.28	d-[(MeO) <sub>2</sub> Tr]ibuGpibuGpTOAc (0.0153)	22
d-[(MeO) <sub>2</sub> Tr]bzApbzAp (0.065)	d-bzAOAc (0.043)	0.163	d-[(MeO) <sub>2</sub> Tr]bzApbzApbzAOAc (0.023)	53
d-[(MeO) <sub>2</sub> Tr]bzApbzAp (0.13)	d-bzApbzAOAc (0.20)	0.52	d-[(MeO) <sub>2</sub> Tr]bzApbzApbzApbzAOAc (0.031)	24
d-[(MeO) <sub>2</sub> Tr]bzApbzAp (0.0168)	d-ibuGpzbAOAc (0.0168)	0.0735	d-[(MeO) <sub>2</sub> Tr]ApbzApibuGpzbAOAc (0.0029)	17

Table II: Ultraviolet Spectral Properties and Chromatographic Mobilities of Protected Oligodeoxyribonucleoside Methylphosphonates

oligomer	UV spectra <sup>a</sup>						silica gel TLC R <sub>f</sub> <sup>b</sup> in MeOH-CHCl <sub>3</sub>				silica gel HPLC <sup>c</sup> retention time (min)
	$\lambda_{\max}$ (nm)	$\lambda_{\min}$ (nm)	$\epsilon_{260}/\epsilon_{235}$		$\epsilon_{260}/\epsilon_{280}$		5%	10%	15%	20%	
			calcd	obsd	calcd	obsd					
d-[(MeO)Tr]TpTpTpTpCNEt	265 235 sh	243	1.34	1.31	1.55	1.64	—	—	0.08	0.29	—
d-[(MeO)Tr]Tp(Tp) <sub>2</sub> TPCNEt	265	243	1.75	0.92	1.57	1.56	—	0.00	—	0.13	—
d-[(MeO) <sub>2</sub> Tr]ibuGpibuGpCNEt	238 253 260 280	225 245 256 270	1.19	1.05	1.33	1.32	—	0.16	—	—	19.2
d-[(MeO) <sub>2</sub> Tr]ibuGpzbAOAc	235 278	256	0.82	0.75	0.88	0.87	—	0.29	—	—	12.3
d-ibuGpzbAOAc	260 280	239 267	1.63	1.27	0.90	0.90	—	0.18 0.14	—	—	15.5 17.6
d-[(MeO) <sub>2</sub> Tr]ibuGpibuGpTOAc	240 sh 260 275 sh	228	1.34	1.51	1.38	1.45	—	0.18	—	—	16.0
d-[(MeO) <sub>2</sub> Tr]bzApbzApbzAOAc	234 280	227 255	0.66	0.61	0.59	0.59	—	0.41 0.38	0.55 0.53	—	13.4 14.3
d-[(MeO) <sub>2</sub> Tr]bzApbzApbzApbzAOAc	233 sh 280	253	0.71	0.60	0.59	0.60	—	—	0.31	—	19.3
d-[(MeO) <sub>2</sub> Tr]bzApbzApibuGpzbAOAc	235 sh 280	255	0.89	0.74	0.73	0.75	—	0.15	0.44	—	23.8

<sup>a</sup> Measured in 95% EtOH. <sup>b</sup> E. Merck silica gel 60 F<sub>254</sub> sheets, 0.2 mm thick. <sup>c</sup> HC Pellosil (2.1 mm x 1 m); 0-20% methanol in chloroform; 1 mL/min; 40-mL total volume.

by transformed Syrian hamster fibroblasts was determined as previously described (Miller et al., 1977).

**Effects of Oligodeoxyribonucleoside Methylphosphonates on Colony Formation.** (1) *E. coli*. *E. coli* B was grown in M-9 medium (Bolle et al., 1968) supplemented with glucose (36 g/L) and 1% casamino acids. The cells were harvested in mid-log phase and resuspended in 50  $\mu$ L of fresh medium containing 0-160  $\mu$ M oligomer at a final cell density of  $1 \times 10^4$  cells/mL. The cells were incubated for 1 h at 37 °C and then diluted with 0.9 mL of medium. A 0.8-mL aliquot was added to 2.5 mL of 0.8% Bactoagar at 45 °C. This solution was quickly poured onto a 100-mm plate containing solid 1.2% Bactoagar. After solidification, the plates were incubated overnight at 37 °C and the resulting colonies were counted.

(2) *Transformed Syrian Hamster Embryonic Fibroblasts (BP-6) and Transformed Human Fibroblasts (HTBI080)*. Colony formation by the fibroblasts in the presence of the methylphosphonate analogues was carried out as previously described (Miller et al., 1977).

## Results

**Synthesis of Oligodeoxyribonucleoside Methylphosphonates.** The synthetic scheme used for preparing the oligonucleoside methylphosphonates followed the basic approach used to synthesize dideoxyribonucleoside methylphosphonates (Miller et al., 1979). Suitably protected monomers or oligomer blocks carrying a 3'-terminal methylphosphonate group were condensed with protected mono- or oligonucleotides bearing a free 5'-hydroxyl group. Mesityl-

enesulfonyl tetrazolide (Stawinsky et al., 1977) was used as the condensing agent. The fully protected oligomers were purified by silica gel column chromatography. The reaction conditions used and the yields obtained are given in Table I. The oligomers were characterized by ultraviolet spectroscopy, thin-layer chromatography, and high-pressure liquid chromatography as indicated in Table II.

The protecting groups were removed as previously described (Miller et al., 1979). In the case of the deoxyadenosine-containing oligomers, the *N*-benzoyl groups were first removed by treatment with hydrazine hydrate (Letsinger et al., 1968). The remaining 3'-*O*-acetyl and 5'-*O*-dimethoxytrityl groups were removed by sequential treatment with ammonium hydroxide and 80% acetic acid. The oligomers were purified by preparative paper chromatography and were characterized by UV spectroscopy (Table III).

**Interaction of Oligodeoxyribonucleoside Methylphosphonates with Complementary Polynucleotides.** Table IV summarizes the melting temperatures of complexes formed between oligodeoxyadenosine methylphosphonates and poly(U) or poly(dT). For comparison, the melting temperatures of complexes formed by oligodeoxyribo- and oligoriboadenosines are included. Each oligomer forms a triple-stranded complex with a stoichiometry of 2U:1A or 2T:1A. The melting temperatures increase as the chain length of the oligonucleotide increases. For a given chain length, the complexes formed by the methylphosphonate analogues melt at higher temperatures than those formed by the natural diester oligomers. With the exception of r-ApApApA, the complexes formed by the oli-

## NONIONIC NUCLEIC ACID METHYLPHOSPHONATES

Table III: Spectral Properties and Chromatographic Mobilities of Oligodeoxyribonucleoside Methylphosphonates

oligomer	UV spectra <sup>a</sup>				paper chromatography <sup>b</sup> $R_f$ , solvent A
	$\lambda_{\max}$ (nm)	$\lambda_{\min}$ (nm)	$\epsilon_{260}/\epsilon_{280}$	$\epsilon$ at $\lambda_{\max}$	
d-GpGpT <sup>c</sup>	257	230	1.45	$33.4 \times 10^3$	0.31
	270 sh				
d-ApApA	258	232	4.27	$39.0 \times 10^3$	0.29
d-ApApApA	258	230	3.77	$50.4 \times 10^3$	0.11
d-ApApGpA	258	227	3.03	$50.3 \times 10^3$	0.11
d-Tp[ <sup>3</sup> H]T	267	235	1.53		0.59
d-(Tp) <sub>4</sub> [ <sup>3</sup> H]T	266	235	1.49		0.21
d-(Tp) <sub>8</sub> [ <sup>3</sup> H]T	266	235	1.56		0.17

<sup>a</sup> Measured in water, pH 7.0. <sup>b</sup>  $R_f$  of pT = 0.11. <sup>c</sup> The UV spectrum is similar to that of d-GpGpT (Miller et al., 1974).

Table IV: Interaction of Oligonucleoside Methylphosphonates with Complementary Polynucleotides<sup>a</sup>

oligomer	$T_m$ with poly(U) (2U:1A) (°C)	$T_m$ with poly(dT) (2T:1A) (°C)
d-ApA: isomer 1	15.4	18.7
isomer 2	19.8	18.4
d-ApApA	33.0	36.8
d-ApApApA	43.0	44.5
d-ApA	7.0	9.2
d-ApApApA	32.0	35.5
r-ApApApA	36.2	2.4

<sup>a</sup>  $5 \times 10^{-5}$  M total [nucleotide], 10 mM Tris, and 10 mM MgCl<sub>2</sub>, pH 7.5.

omers with poly(dT) have slightly higher melting temperatures than the corresponding complexes formed with poly(U).

The interaction of d-GpGp[<sup>3</sup>H]T with unfractionated tRNA<sub>E.coli</sub> was measured by equilibrium dialysis (Miller et al., 1974). The apparent association constants at 0, 22, and 37 °C are 1100 M<sup>-1</sup>, 200 M<sup>-1</sup>, and 100 M<sup>-1</sup>, respectively. These binding constants are much lower than those of the 2'-O-methylribooligonucleotide ethyl phosphotriester, G<sup>m</sup>(Et)-G<sup>m</sup>(Et)[<sup>3</sup>H]U, which are 9300 M<sup>-1</sup> (0 °C), 1900 M<sup>-1</sup> (22 °C), and 2000 M<sup>-1</sup> (37 °C) (Miller et al., 1977).

**Effect of Oligodeoxyribonucleoside Methylphosphonates on Cell-Free Aminoacylation of tRNA.** The effects of selected oligodeoxyribonucleoside methylphosphonates on aminoacylation of unfractionated tRNA<sub>E.coli</sub> are shown in Table V. Three amino acids were tested at various temperatures. The deoxyadenosine-containing analogues which are complementary to the -UUU- sequence of the anticodon of tRNA<sub>E.coli</sub> have the largest inhibitory effect on aminoacylation of tRNA<sub>E.coli</sub>. The percent inhibition increases with increasing chain length and decreases with increasing temperature. Inhibition by d-ApApGpA and by the diesters d-ApApApA and r-ApApApA is less than that exhibited by d-ApApApA. In contrast to their behavior with tRNA<sub>E.coli</sub>, neither the methylphosphonates, d-ApApApA and d-ApApGpA, nor the phosphodiester, d-ApApApA and r-ApApApA, had any inhibitory effect on tRNA<sub>rabbit</sub> in the rabbit reticulocyte cell-free system (data not shown).

**Effects of Oligodeoxyribonucleoside Methylphosphonates on Cell-Free Protein Synthesis.** The ability of deoxyadenosine-containing oligonucleoside methylphosphonates to inhibit polypeptide synthesis in cell-free systems directed by synthetic and natural messages was tested. The results of these experiments are given in Table VI. Poly(U)-directed phenylalanine incorporation and poly(A)-directed lysine incorporation are both inhibited by oligodeoxyadenosine methylphosphonates and diesters in the *E. coli* system at 22 °C. The percent inhibition increases with oligomer chain length and is greater for polyphenylalanine synthesis. The methylphosphonate analogues are more effective inhibitors than either

Table V: Effects of Oligonucleoside Methylphosphonates on Aminoacylation in an *E. coli* Cell-Free System

oligomer <sup>a</sup>	% inhibition <sup>b</sup>				
	Phe, 0 °C	Leu, 0 °C	Lys		
			0 °C	22 °C	37 °C
d-ApA	6	0	7		
d-ApApA	9	0	62	15	0
d-ApApApA	9	12	88	40	16
d-ApApGpA	12	12	35	0	
d-GpGpT	31	5	34	9	15
dGpGpT (400 μM)	23				
d-ApApApA	0	7	71 <sup>c</sup>	15 <sup>c</sup>	
r-ApApApA			78 <sup>d</sup>	17 <sup>d</sup>	

<sup>a</sup> [oligomer] = 50 μM. <sup>b</sup> [tRNA<sub>E.coli</sub>] = 2 μM. <sup>c</sup> [oligomer] = 100 μM. <sup>d</sup> [oligomer] = 125 μM.

Table VI: Effects of Oligonucleoside Methylphosphonates on Bacterial and Mammalian Cell-Free Protein Synthesis at 22 °C

oligomer	% inhibition			
	<i>E. coli</i>		rabbit reticulocyte	
	poly(U) directed <sup>a</sup>	poly(A) directed <sup>b</sup>	poly(U) directed <sup>a</sup>	globin mRNA directed <sup>c</sup>
d-ApA	20	10		
d-ApApA	84	30	81	
d-ApApApA	100	65	77	0
d-ApApGpA	22			0
d-ApApApA	13	19	18	0
r-ApApApA	18	17	85	0

<sup>a</sup> [poly(U)] = 360 μM in U; [oligomer] = 175–200 μM in base.  
<sup>b</sup> [poly(A)] = 300 μM in A; [oligomer] = 175–200 μM in base.  
<sup>c</sup> [oligomer] = 200 μM in base.

d-ApApApA or r-ApApApA at the same concentration. Although both the oligodeoxyadenosine methylphosphonates and the phosphodiester inhibit translation of poly(U) in the rabbit reticulocyte system, no effect on the translation of globin message was observed. As in the case of the *E. coli* system, inhibition of phenylalanine incorporation increased with oligomer chain length and was greater for the methylphosphonate analogues than for the diesters.

**Uptake of Oligodeoxyribonucleoside Methylphosphonates by Mammalian Cells.** Figure 1 shows the incorporation of radioactive 100 μM d-GpGp[<sup>3</sup>H]T with time into transformed Syrian hamster embryonic fibroblasts growing in monolayer. The incorporation is approximately linear for the first hour and begins to level off after 1.5 h. The concentration of radioactivity inside the cells is ~117 μM after 1.5 h assuming a cell volume of 1.5 μL/10<sup>6</sup> cells (Hempling, 1972).

Cells were incubated with 25 μM d-GpGp[<sup>3</sup>H]T for 18 h. The medium was removed, and the cells were washed with phosphate buffer and then lysed with NaDodSO<sub>4</sub>. Approx-

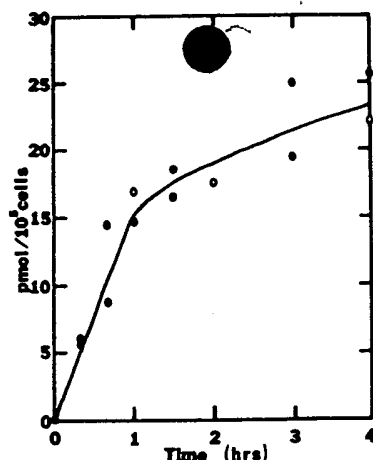


FIGURE 1: Transport of (O) 100  $\mu$ M d-GpGp[ $^3$ H]T and (●) 100  $\mu$ M d-(Tp) $_8$ [ $^3$ H]T into transformed Syrian hamster fibroblasts growing in monolayer at 37  $^{\circ}$ C.

imately 30% of the total radioactivity from the lysate was found in  $\text{Cl}_3\text{AcOH}$ -precipitable material. The DNA was precipitated from the lysate and digested with deoxyribonuclease and snake venom phosphodiesterase. The culture medium, the DNA-free lysate, and the DNA digest were each examined by paper chromatography. Only intact d-GpGp[ $^3$ H]T was found in the medium. Radioactivity corresponding to [ $^3$ H]TTP (6%) and to d-GpGp[ $^3$ H]T (94%) was found in the lysate, while the DNA digest gave [ $^3$ H]dpT and [ $^3$ H]dT as products.

Similar uptake studies were carried out with d-AP[ $^3$ H]T and with a series of oligothymidylate analogues, d-(Tp) $_n$ [ $^3$ H]T ( $n = 1, 4, \text{ and } 8$ ). The rates and extents of uptake of these analogues were very similar to that of d-GpGp[ $^3$ H]T (Figure 1). Examination of the culture medium and cell lysate after overnight incubation with these oligonucleotides gave results similar to those found for d-GpGp[ $^3$ H]T.

**Effects of Oligodeoxyribonucleoside Methylphosphonates on Colony Formation by Bacterial and Mammalian Cells.** The effects of selected oligodeoxyribonucleoside methylphosphonates on colony formation by *E. coli* B, transformed Syrian hamster fibroblast (BP-6), and transformed human fibroblast (HTB 1080) cells are summarized in Table VII. The d-(Ap) $_n$ A analogues appear to inhibit *E. coli* colony formation at high concentrations (160  $\mu$ M). However, no inhibitory effects on the incorporation of [ $^3$ H]leucine into cellular protein or [ $^3$ H]thymidine into cellular DNA could be detected in the presence of these compounds.

Colony formation of both transformed hamster and human cells are inhibited to various extents by the oligonucleoside methylphosphonates. Both the hamster and human cells appear to be affected to a similar extent by a given analogue. It appears in the case of d-AP $_n$ A that each diastereoisomer exerts a different inhibitory effect on the growth of the hamster cells. As in the case of *E. coli*, no inhibition of cellular protein synthesis could be detected.

## Discussion

Oligodeoxyribonucleoside methylphosphonates with sequences complementary to the anticodon loop of tRNA $^{Lys}$  and to the -ACCA-OH amino acid accepting stem of tRNA were prepared in a manner similar to that used to prepare dideoxyribonucleoside methylphosphonates (Miller et al., 1979). The present studies demonstrate the ability to join blocks of protected methylphosphonates to give oligomers with chain lengths up to nine nucleotidyl units. The yields in these

Table VII: Effects of Oligonucleoside Methylphosphonates on Colony Formation by Bacterial and Mammalian Cells in Culture

oligomer	% inhibition <sup>a</sup>			
	<i>E. coli</i> B			
	50 $\mu$ M	160 $\mu$ M	BP-6, 50 $\mu$ M	HTB 1080, 50 $\mu$ M
d-AP $_n$ T	4	5	5, 16 <sup>b</sup>	12
d-AP $_n$ A	8	58	6, <1 <sup>b</sup>	5
d-AP $_n$ ApA	3	44	29	31
d-AP $_n$ ApApA	19	78	36	19
d-GpGpT	7	11	7	9

<sup>a</sup> The results are the average of two or three experiments. Each experiment consisted of two plates (bacterial cells) or three plates (mammalian cells). The average variation is  $\pm 3\%$  in percent inhibition. The cells were treated with and grown in the presence of the oligomer at 37  $^{\circ}$ C. <sup>b</sup> The percent inhibition of isomers 1 and 2, respectively.

condensation reactions are acceptable, although reactions involving deoxyguanosine residues appear to proceed in low yield. Similar difficulties have been encountered in the syntheses of oligonucleotide phosphotriesters. Unlike the dideoxyribonucleoside methylphosphonates previously reported, the oligodeoxyribonucleoside methylphosphonates prepared for this study were not resolved into their individual diastereoisomers.

The oligodeoxyadenosine analogues form triple-stranded complexes with both poly(U) and poly(dT). These complexes are more stable than similar complexes formed by either oligoribo- or oligodeoxyribonucleotides. As previously suggested for oligonucleotide ethyl phosphotriesters (Miller et al., 1971, 1974; Pless & Ts'o, 1977) and dideoxyribonucleoside methylphosphonates (Miller et al., 1979), this increased stability is attributed to the decreased charge repulsion between the nonionic backbone of the analogue and the negatively charged complementary polynucleotide backbone. With the exception of r-AP $_n$ ApA (Table IV), the stability of the complexes formed with poly(dT) are slightly higher than those formed with poly(U), a situation which is also observed for the interaction of poly(dA) with poly(dT) and with poly(U) (Chamberlin, 1965). The lower stability of the (r-AP $_n$ ApA)-2[poly(dT)] complex is also reflected at the polymer level. Thus, under the conditions of the experiments described in Table IV, we found that the  $T_m$  of poly(rA)-2[poly(rU)] is 83  $^{\circ}$ C while the  $T_m$  of poly(rA)-2[poly(dT)] is 59  $^{\circ}$ C. These results are consistent with those of Riley et al. (1966). They observed that formation of the poly(rA)-2[poly(dT)] complex occurs only at a sodium ion concentration of 2.5 M in the absence of magnesium, while poly(rA)-2[poly(rU)] forms in 0.1 M sodium phosphate buffer.

The oligodeoxyadenosine methylphosphonates and their parent diesters selectively inhibit cell-free aminoacylation of tRNA $^{Lys}_{E.coli}$ . The extent of inhibition is temperature dependent and parallels the ability of the oligomers to bind to poly(U). These observations and the previously demonstrated interaction of r-AP $_n$ ApA with tRNA $^{Lys}_{E.coli}$  (Möller et al., 1978) suggest that inhibition occurs as a result of oligomer binding to the -UUUU- anticodon loop of the tRNA. The reduced inhibition observed with d-AP $_n$ ApGpA is consistent with this explanation, since interaction of this oligomer with the anticodon loop would involve formation of a less stable G-U base pair.

Recent studies by Ramberg et al. (1978) have shown that the rate of aminoacylation of tRNA $^{Lys}_{E.coli}$  substituted with 5-fluorouracil is considerably lower than that of nonsubstituted tRNA $^{Lys}_{E.coli}$ . The increased  $K_m$  of the 5-fluorouracil-substituted tRNA suggested a decreased interaction with the lysyl aminoacyl synthetase. These results and those of Saneyoshi

& Nishimura (1971) suggest that the anticodon loop of tRNA<sup>Lys</sup><sub>E.coli</sub> is part of the synthetase recognition site. Thus, inhibition of aminoacylation by the oligodeoxyribonucleoside methylphosphonates could result from the reduction in the affinity of the synthetase for tRNA<sup>Lys</sup>-oligonucleotide complexes. The greater inhibition observed with d-ApApApA vs. the diesters, d-ApApApA or r-ApApApA, may result from greater binding of the analogue to the anticodon loop or to the decreased ability of the synthetase to displace the nonionic oligonucleotide analogue vs. the phosphodiester oligomers from the anticodon loop. Alternatively, oligomer binding to the anticodon loop could induce a conformational change in the tRNA, leading to a lower rate and extent of aminoacylation. Such conformational changes have been detected when r-ApApApA binds to tRNA<sup>Lys</sup><sub>E.coli</sub> (Möller et al., 1979; Wagner & Garrett, 1979).

None of the oligomers have any effect on the aminoacylation of tRNA<sup>Lys</sup><sub>rabbit</sub> in a cell-free system. Since the anticodon regions of tRNAs from bacterial and mammalian sources probably are similar, the oligo(A) analogues are expected to interact with the anticodon region of both tRNA<sup>Lys</sup>s. The failure to observe inhibition of aminoacylation of tRNA<sup>Lys</sup><sub>rabbit</sub> in the presence of these oligo(d-A) analogues suggests that there may be a difference between the interaction of the lysine aminoacyl synthetase with tRNA<sup>Lys</sup> from *E. coli* and from rabbit systems or a difference between the structure of these two tRNA<sup>Lys</sup>s in response to the binding of oligo(d-A) analogues.

The trimer, dGpGpT, inhibits both phenylalanine and lysine aminoacylation at 0 °C but has little effect on leucine aminoacylation. The aminoacyl stems of both tRNA<sup>Lys</sup><sub>E.coli</sub> and tRNA<sup>Phe</sup><sub>E.coli</sub> terminate in a G-C base pair between nucleotides 1 and 72, while a less stable G-U base pair is found at this position in tRNA<sup>Leu</sup><sub>E.coli</sub> (Sprinzl et al., 1978). Thus the observed differences in inhibition of aminoacylation by d-GpGpT may reflect differences in the ability of this oligomer to bind to the different -ACC- ends of the various tRNAs.

Inhibition of lysine aminoacylation by dGpGpT is very temperature sensitive and parallels the decrease in binding to tRNA with increasing temperature. This behavior of d-GpGpT contrasts that of G<sub>p</sub><sup>m</sup>(Et)G<sub>p</sub><sup>m</sup>(Et)U (Miller et al., 1977). Although both oligomers can potentially interact with the same sequences in tRNA, the 2'-O-methylribotrinucleotide ethyl phosphotriester binds more strongly and more effectively inhibits aminoacylation. The differences in binding ability may be due to overall differences in the conformation of the deoxyribo vs. 2'-O-methylribo backbones of these oligomers.

The oligodeoxyribonucleoside methylphosphonates effectively inhibit polyphenylalanine synthesis in cell-free systems derived from both *E. coli* and rabbit reticulocytes. In the *E. coli* system, the extent of inhibition by the oligodeoxyadenosine analogues parallels the  $T_m$  values of the oligomers with poly(U). The tetramer, d-ApApGpA, which would have to form a G-U base pair with poly(U), was 4.5-fold less effective than d-ApApApA. These results suggest that the oligomers inhibit polypeptide synthesis as a consequence of forming complexes with the poly(U) message. A similar inhibitory effect by poly(dA) on the translation of poly(U) was observed by Williamson et al. (1967). It is unlikely that inhibition results from nonspecific interaction of the methylphosphonates with protein components of the translation systems. In the *E. coli* system, poly(A) translation is inhibited to a lesser extent than is translation of poly(U), while in the reticulocyte system, no inhibition of globin mRNA translation is observed.

The data suggest that the magnitude of inhibition of poly-(U)-directed polypeptide synthesis in the *E. coli* system does

not reflect proportionally the ability of the oligomer to bind to poly(U). Although the oligomer pairs d-ApApA/d-ApApApA and d-ApApApA/r-ApApApA form complexes with poly(U) which have very similar  $T_m$  values (see Table IV), in each case the methylphosphonate analogues inhibit 5.5-6.5 times better than do the diesters. This stronger inhibitory effect could result from a decreased ability of the ribosome to displace the nonionic oligodeoxyribonucleoside methylphosphonates from the poly(U) message, or, alternatively, there may be a degradation of the oligonucleotides (phosphodiester) by nucleases in the cell-free translation systems but not the corresponding phosphonate analogues.

Experiments with radioactively labeled oligonucleotide methylphosphonates show that these analogues are taken up by mammalian cells growing in culture. The extent of uptake is consistent with passive diffusion of the oligomer across the cell membrane. Both d-Tp[<sup>3</sup>H]T and d-(Tp)<sub>8</sub>[<sup>3</sup>H]T are taken up to approximately the same extent, which suggests that there is no size restriction to uptake over this chain length range. This behavior is in contrast to results obtained with *E. coli* B cells (K. Jayaraman et al., unpublished results).

Examination of lysates of mammalian cells exposed to labeled oligomers for 18 h showed that ~70% of the labeled thymidine was associated with intact oligomer with the remainder found in thymidine triphosphate and in cellular DNA. These observations indicate that the oligodeoxyribonucleoside methylphosphonates, which are recovered intact from the culture medium, are slowly degraded within the cell. Failure to observe shorter oligonucleotides and the known resistance of the methylphosphonate linkage to nuclease hydrolysis suggests that degradation may result from cleavage of the 3'-terminal [<sup>3</sup>H]thymidine *N*-glycosyl bond with subsequent reutilization of the thymine base.

The uptake process of the oligonucleoside methylphosphonates is quite different from that of previously studied oligonucleotide ethyl phosphotriesters (Miller et al., 1977; P. S. Miller et al., unpublished results). In the case of G<sub>p</sub><sup>m</sup>(Et)G<sub>p</sub><sup>m</sup>(Et)[<sup>3</sup>H]U, the oligomer is rapidly taken up by the cells and is subsequently deethylated. Further degradation to smaller oligomers is then observed, presumably as a result of nuclease-catalyzed hydrolysis of the resulting phosphodiester linkages. Approximately 80% of the oligomer is metabolized within 24 h. Although the rate of uptake of d-Gp(Et)Gp(Et)[<sup>3</sup>H]T is similar to that of d-GpGp[<sup>3</sup>H]T, examination of the cell lysate showed extensive degradation of the phosphotriester analogue. The relatively long half-lives of the oligodeoxyribonucleoside methylphosphonates may be of value in potential pharmacological applications of these oligonucleotide analogues.

The effects of these analogues on cell colony formation confirmed that the methylphosphonates are taken up by both mammalian and bacterial cells. All the oligomers tested inhibited colony formation of both cell types to various extents. The mechanism(s) by which these compounds exert their inhibitory effects is (are) currently under investigation. No decrease in either overall short-term cellular protein synthesis or DNA synthesis was detected by the present procedure in the presence of these compounds. This does not rule out the possibility that the syntheses of certain critical proteins are perturbed by these oligomers. We are currently studying this possibility by examination of the cellular proteins using two-dimensional gel electrophoresis.

The experiments described in this paper extend our studies on the use of nonionic oligonucleotides as sequence-function probes of nucleic acids both in biochemical experiments and

in living cells. In a future publication we will describe the effects of an oligodeoxynucleoside methylphosphonate complementary to the 3' terminus of 16S rRNA on bacterial protein synthesis and growth (K. Jayaraman et al., unpublished results). Our results suggest that sequence-specific oligonucleoside methylphosphonates may find important applications in probing and regulating nucleic acid function within living cells.

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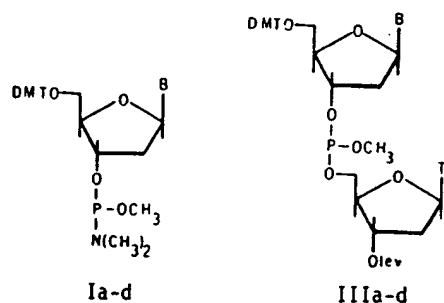
# DEOXYNUCLEOSIDE PHOSPHORAMIDITES—A NEW CLASS OF KEY INTERMEDIATES FOR DEOXPOLYNUCLEOTIDE SYNTHESIS

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The development of a new class of nucleoside phosphites is described. These compounds are stable to normal laboratory conditions, are activated by mild acid treatment, and are observed to react essentially quantitatively with protected nucleosides.

A recent, key innovation in oligonucleotide synthesis was the introduction of the phosphite coupling approach by Letsinger and coworkers (1-3). This approach has been adapted to the synthesis of deoxyoligonucleotides (4-8), oligoribonucleotides (9-12), and nucleic acid analogs (13-15). Generally the approach involves the reaction of a suitably protected nucleoside, a bifunctional phosphitylating agent such as methoxydichlorophosphine, and a second protected nucleoside. Mild oxidation using iodine in tetrahydrofuran, lutidine and water generates the natural internucleotide bond. By varying the oxidation procedure, phosphorus analogs such as selenophosphates (14), imidophosphates (14) and thiophosphates (14, 15) can be generated. A serious limitation of this methodology, however, has been the instability of the reactive intermediates (nucleoside phosphomonochloridites or monotetrazolides) towards hydrolysis and air oxidation. This problem has been circumvented by either preparing the reactive species immediately prior to use or storing the active phosphite as a precipitate in hexanes at -20°C. We have recently solved this problem by synthesizing a new class of nucleoside phosphites that are easy to prepare by standard organochemical procedures, are stable under normal laboratory conditions to hydrolysis and air oxidation, and are stored as dry, stable powders. These key intermediates are N, N-dimethylaminophosphoramidites of the appropriately protected deoxynucleosides and are



Ia, IIIa, B = 1-Thymine  
 Ib, IIIb, B = 1-(N-4-Benzoylcytosine)  
 Ic, IIIc, B = 9-(N-6-Benzoyladenine)  
 Id, IIId, B = 9-(N-2-Isobutyrylguanine)  
 lev = levulinyl  
 DMT = Di-p-anisylphenylmethyl

represented as compounds Ia-d. This communication outlines the synthesis, characterization, and reactivity of these phosphoramidites.

The synthesis of compounds Ia-d begins with the preparation of chloro-N, N-dimethylamino-methoxyphosphine [ $\text{CH}_3\text{O P}(\text{Cl}) \text{N}(\text{CH}_3)_2$ ] which is used as a monofunctional phosphitylating agent. A 250 ml addition funnel was charged with 100 ml of precooled anhydrous ether (-78°C) and precooled (-78°C) anhydrous dimethylamine (45.9 g, 1.02 mol). The addition funnel was wrapped with aluminum foil containing dry ice in order to avoid evaporation of dimethylamine. This



solution was added dropwise at  $-15^{\circ}\text{C}$  (ice-acetone bath) over 2 h to a mechanically stirred solution of methoxydichlorophosphine (16) (47.7 ml, 67.32 g., 0.51 mol) in 300 ml of anhydrous ether. The addition funnel was removed and the 1 L, three-necked round bottom flask was stoppered with serum caps tightened with copper wire. The suspension was mechanically stirred for 2 h at room temperature. The suspension was filtered and the amine hydrochloride salt was washed with 500 ml anhydrous ether. The filtrate and washings were combined and ether was distilled at atmospheric pressure. The residue was distilled under reduced pressure. The product was collected at  $40-42^{\circ}\text{C}$  @ 13 mm Hg and was isolated in 71% yield (51.1 g, 0.36 mol).  $d_{25}^{25} = 1.115 \text{ g/ml}$ .  $^{31}\text{P}$ -N.M.R.,  $\delta = -179.5 \text{ ppm}$  ( $\text{CDCl}_3$ ) with respect to internal 5% v/v aqueous  $\text{H}_3\text{PO}_4$  standard.  $^1\text{H}$ -N.M.R. doublet at 3.8 and 3.6 ppm  $J_{\text{P-H}} = 14 \text{ Hz}$  (3H,  $\text{OCH}_3$ ) and two singlets at 2.8 and 2.6 ppm (6H,  $\text{N}(\text{CH}_3)_2$ ). The mass spectrum showed a parent peak at  $m/e = 141$ .

The key intermediates Ia-d were prepared by the following procedure. 5'-O-Di-*p*-anisyl phenylmethyl nucleoside (1 mmol) was dissolved in 3 ml of dry, acid free chloroform and diisopropylethylamine (4 mmol) in a 10 ml reaction vessel preflushed with dry nitrogen.  $[\text{CH}_3\text{OP}(\text{Cl})\text{N}(\text{CH}_3)_2]$  (2 mmol) was added dropwise (30-60 sec) by syringe to the solution under nitrogen at room temperature. After 15 min the solution was transferred with 35 ml of ethyl acetate into a 125 ml separatory funnel. The solution was extracted four times with an aqueous saturated solution of NaCl (80 ml). The organic phase was dried over anhydrous  $\text{Na}_2\text{SO}_4$  and evaporated to a foam under reduced pressure. The foam was dissolved with toluene (10 ml) (Id was dissolved with 10 ml of ethyl acetate) and the solution was added dropwise to 50 ml of cold hexanes ( $-78^{\circ}\text{C}$ ) with vigorous stirring. The cold suspension was filtered and the white powder was washed with 75 ml of cold hexanes ( $-78^{\circ}\text{C}$ ). The white powder was dried under reduced pressure and stored under nitrogen. Isolated yields of compounds Ia-d were 90-94% (see Table I).

TABLE I

COMPOUND	$\delta\text{-}^{31}\text{P}$ (ppm) (Acetone- $d_6$ )	$\delta\text{-}^{31}\text{P}$ (ppm) ( $\text{CDCl}_3$ )	ISOLATED YIELD (%)
Ia	-146.0, -145.4	-147.7, -146.8	93, 95*
Ib	-146.3, -145.5	-148.0, -147.0	92, 95*
Ic	-146.1, -145.8	-147.4, -147.3	90, 98*
Id	-145.9, -145.7	-147.7, -147.2	90, 98*
IIa	-139.6, -138.9	-140.8, -139.9	97**
IIb	-139.6, -139.0	-140.6, -140.0	94**
IIc	-139.7, -138.9	-141.0, -139.9	97**
IIId	-140.3, -140.2	-143.6, -141.9	93**

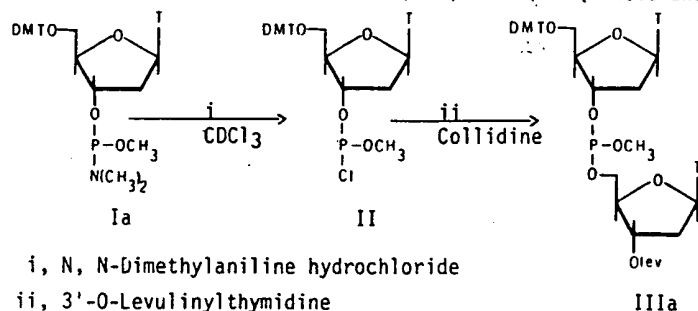
\*Estimated purity from  $^{31}\text{P}$ -N.M.R.

\*\*Estimated yield from  $^{31}\text{P}$ -N.M.R.

The purity of the products was checked by  $^{31}\text{P}$ -N.M.R. Additionally, when analyzed by  $^{31}\text{P}$ -N.M.R. these compounds were stable for at least a month when stored at room temperature under nitrogen. Furthermore, no significant amount of 3'-3' dinucleoside phosphite was detected by  $^{31}\text{P}$ -N.M.R. (less than 4%). The low content of the 3'-3' dinucleoside phosphite was expected and represented a significant improvement over the original phosphite coupling procedure where a considerable amount of the unwanted 3'-3' dinucleoside phosphite was unavoidable (1-3, 9).

We have observed that mild acidic conditions can be used to activate Ia-d toward formation

of phosphite internucleotide bonds. These investigations were prompted by earlier research showing that aminophosphines can be protonated and therefore activated by acidic species (17-24). This activation process was initially monitored by  $^{31}\text{P}$ -N.M.R. Thus, when *N,N*-dimethylaniline hydrochloride (1 mmol) in 0.5 ml of dry  $\text{CDCl}_3$  was added at room temperature under nitrogen to Ia (0.5 mmol, -147.7 and -146.8 ppm) in 2 ml of dry, acid free  $\text{CDCl}_3$  in a 10 mm N.M.R. tube, the chlorophosphite II (-167.2 ppm) was obtained in quantitative yield. Addition of 1.2 molar equivalents of 3'-O-levulinylthymidine (25) to the chlorophosphite II led to an essentially quantitative conversion to the dinucleoside phosphite IIIa (-140.8 and -139.9 ppm).



Evidence supporting the assignment of the active chlorophosphite II to the peak at -167.2 was independently obtained by reacting 5'-*O*-Di-*p*-anisylphenylmethylthymidine with excess methoxydi-chlorophosphine (-181.6 ppm) in the presence of collidine in  $\text{CDCl}_3$ . The major reaction product as monitored by  $^{31}\text{P}$ -N.M.R. was localized at -167.2 ppm.

Of the various weak acids investigated as potential activating agents, 1H-tetrazole fulfills all requirements. The compound is a non-hygroscopic, commercially available solid that can be easily purified and dried in one step by sublimation at 110°C @ 0.05 mm Hg. Activation by 1H-tetrazole was also monitored by  $^{31}\text{P}$ -N.M.R. Thus, Ia (0.5 mmol) and 3'-O-levulinylthymidine (0.6 mmol) were placed in a 10 mm N.M.R. tube and sublimed 1H-tetrazole (1.5 mmol) in 2.5 ml of dry acetonitrile- $d_3$  was added under nitrogen atmosphere. The  $^{31}\text{P}$ -N.M.R. spectrum was immediately recorded and displayed a quantitative yield of IIIa. Similar results were also obtained when Ib, Ic and Id were reacted with 3'-O-levulinylthymidine. The appropriate chemical shifts of compounds Ia-d and IIIa-d with respect to internal 5% v/v aqueous  $\text{H}_3\text{PO}_4$  standard are reported in Table I. Complete physical and analytical properties of these compounds will be reported elsewhere.

The applicability of these reagents to the synthesis of deoxyoligonucleotides on polymer supports was also tested. Trial experiments were completed by condensing compounds Ia-d with *N*-2-isobutyryldeoxyguanosine attached covalently to silica gel. Thus, *N*-2-isobutyryldeoxyguanosine (1  $\mu\text{mole}$ ) covalently attached to silica gel (20 mg) at the 3'-position, Ia (10  $\mu\text{mole}$ ), and 1H-tetrazole (50  $\mu\text{mole}$  in 0.1 ml dry acetonitrile) were shaken for 20 min and the reaction was then quenched with aqueous lutidine. The same reaction sequence was completed with Ib, Ic and Id. After the usual oxidation and deprotection procedures (8), d(TpG), d(CpG), d(ApG) and d(GpG) were obtained in 100%, 98%, 94%, and 93% yield respectively (measured spectrometrically from the dimethoxytrityl cation using an extinction of  $7 \times 10^4$  at 498 nm). These dinucleotides were completely degraded by snake venom phosphodiesterase and the appropriate nucleosides and



nucleotides were obtained in the proper ratios (monitored via high pressure liquid chromatography analysis of snake venom phosphodiesterase hydrolysates).

The N, N-dimethylamino phosphines Ia-d therefore display tremendous potential in oligodeoxynucleotide synthesis. These compounds are easy to prepare and are stable to normal laboratory conditions. They are readily activated via protonation and condense with appropriate nucleosides to form internucleotide bonds in very high yields.

#### Acknowledgements

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Solid-phase synthesis of polynucleotides. III. Synthesis of polynucleotides with defined sequences by the block coupling phosphotriester method

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ABSTRACT

Preparation of the three hexadecanucleotides, dGpTpApTpCpAp-CpGpApGpGpCpCpTpT, dCpGpApCpGpApGpCpGpTpGpApCpApCpC and cTpGpCpCpGpGpCpCpApCpGpApTpGpCpG, is described by a rapid and simple solid-phase method on polyacrylamide supports. The syntheses were performed by the extension of the method described in the previous paper using di and trinucleotides of defined sequences as an incoming 3'-phosphodiester unit. Although the coupling yields to form phosphotriester bonds are slightly lower than those for the homothymidylic acid series, pure polydeoxy-ribonucleotides of defined sequences can be synthesized without any major difficulty.

INTRODUCTION

In the previous paper<sup>1</sup>, we reported a rapid and simple solid-phase method for the synthesis of polythymidylic acids. Generally speaking, the yields of the coupling reaction to make internucleotidic phosphotriester bonds between thymidine derivatives are always higher than those among other bases, including guanine, adenine and cytosine, by the solution method. Therefore, the true value of a new approach for the synthesis must always be tested with the synthesis of polynucleotides with defined sequences.

Sequence-specific oligodeoxyribonucleotides are an essential requirement in the various studies of molecular biology; such as gene synthesis for the production of peptide hormones in *E. coli*<sup>2,3</sup>, DNA and RNA sequencing<sup>4,5</sup>, specific probes for hybridization<sup>6</sup>, and so on<sup>7</sup>. All of these studies require the rapid synthesis of oligonucleotides (~ pentadecanucleotide) with defined sequences. The current phosphotriester approach in solution is reasonably rapid and accurate for the synthesis of those oligo-

nucleotides. However, there still remains some problems in the synthesis. One of these problems is the time-consuming chromatographical purification step. In this article, we will report a rapid and accurate solid-phase method for the synthesis of sequence-specific polydeoxyribonucleotides.

#### RESULTS AND DISCUSSION

The chemical principles involved in the synthesis of sequence-specific polydeoxyribonucleotides have been reported in the previous paper<sup>1</sup>. The essential features of the approach are a) sequential addition of di and trinucleotides as coupling units instead of mononucleotides, b) the use of polyacrylamide resin, well-swollen in various solvents and c) the extension of the oligomer chain from the 3'- to the 5'-end. In addition to these features, several modifications have been introduced in this study:

- 1) The unreacted 5'-hydroxyl groups on a solid-phase were completely masked before the next step.
- 2) To avoid depurination of the N-benzoyl deoxyadenosine, the reaction time of the acid treatment to remove dimethoxytrityl groups was shortened to 1 minute at room temperature.
- 3) In addition to the Permaphase AAX column<sup>3</sup>, a  $\mu$ Bondapak C<sub>18</sub> column<sup>8</sup> was used for the final analysis and purification of the products.
- 4) As a protecting group for internucleotidic phosphate bonds, an o-chlorophenyl group was used in place of p-chlorophenyl because the former gave less by-products during the deblocking<sup>9</sup>.

#### Polymer Support and Nucleotide-Polymer Linkage

As described in the accompanying paper<sup>1</sup>, the polymer-support (II) derivatized from the commercially available Enzacryl Gel K-2 (I) has essential advantages over the carboxyl polymer (III) and, therefore, II was used exclusively in this study (Figure 1). The activated esters (IV bcd) were coupled with the resin (II) to afford the dimethoxytrityl resin (V) with a similar yield as in the case of the thymidine base (IVa, 0.15~0.18 mmole/g of nucleoside). The amount of nucleosides attached on the polymer (V) were estimated by the quantitative analysis of dimethoxytrityl group and nucleosides liberated from the resin.

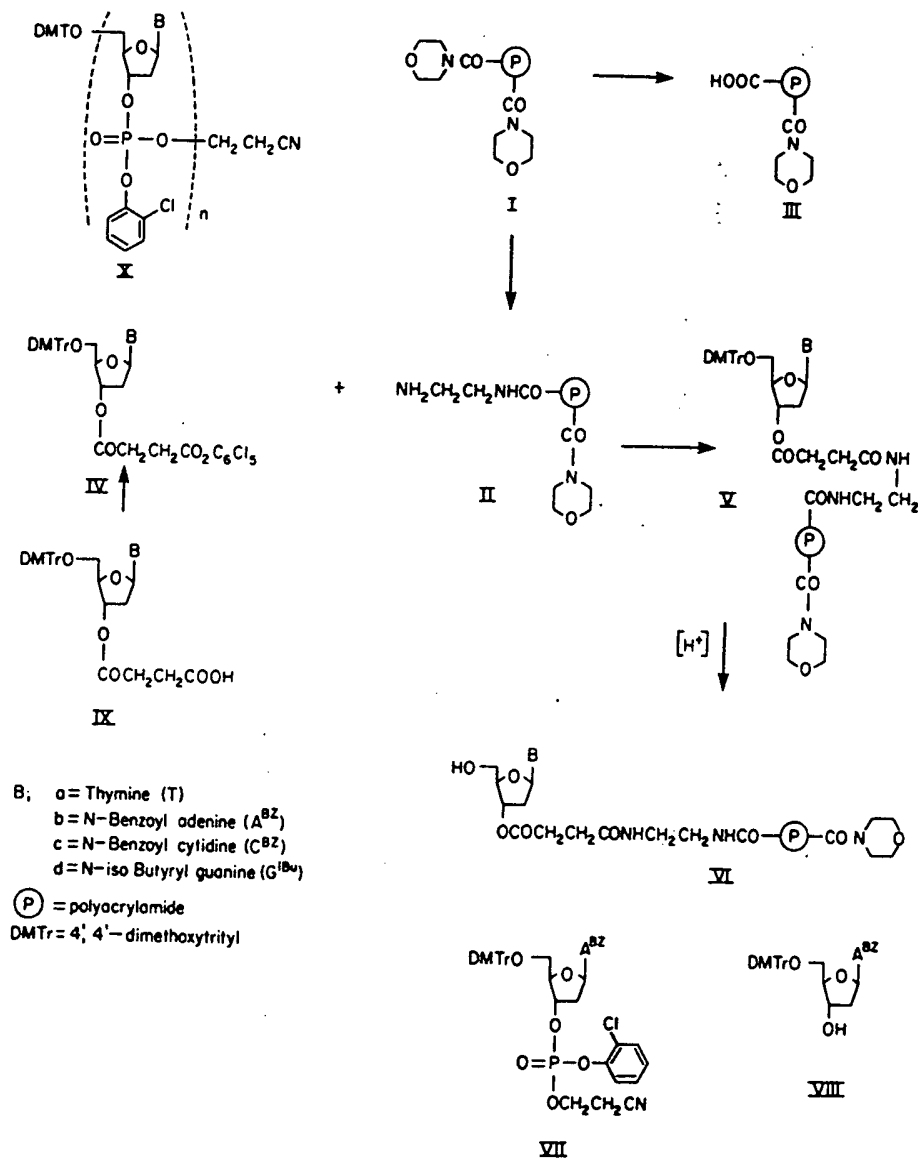


FIGURE 1. Preparation of Resin with the First Nucleoside.

Detritylation of Vb (B = N-benzoyl adenine) under the conditions of a 2% solution of benzenesulfonic acid in chloroform-methanol (7:3 v/v) at room temperature for 1 minute, caused the

depurination (about 10%). It is known that the rate of depurination is dependent upon the substitution group on the 3'-hydroxyl group of the sugar part<sup>10</sup>. Indeed, detritylation of the fully protected mononucleotide (VII), under the conditions described above, does not cause any detectable depurination. On the other hand, N-benzoyl-5'-O-dimethoxytrityl deoxyadenosine (VIII) gives about 15% of N-benzoyl adenine under the same acidic treatment<sup>11</sup>. Accordingly, the one limitation of our solid-phase synthesis using the acid labile protecting group, 4',4'-dimethoxytrityl function, is the synthesis of the polydeoxyribonucleotides with adenine base at the 3'-terminal position. In the solution approach, we observed the same problem and carefully avoided the synthesis of oligonucleotides whose 3'-terminal base sequence was adenine<sup>12</sup>. Actually, this limitation has not been at all serious for the studies of biological problems, because careful design of the sequence does not necessarily require an adenine base at the 3'-terminus position of oligonucleotides. The following methods are suggested to overcome this problem: 1) Development of selective conditions to remove the dimethoxytrityl group. Very recently, two groups (M.H. Caruthers and H. Köster) have found, independently, a new condition to selectively remove the dimethoxytrityl function.<sup>13</sup> 2) Utilization of a new protecting group<sup>14</sup> for the 5'-hydroxyl function, which can be removed under very mild conditions, in place of the dimethoxytrityl group. 3) Utilization of a 3'-phosphotriester bond, such as a phosphoamide, instead of an ester bond for the linkage between the 3'-terminus hydroxyl group of N-benzoyl deoxyadenosine and the polymer support.

#### Masking of the Unreacted 5'-Hydroxyl Group

Quantitative internucleotidic coupling is rarely achieved in oligonucleotide synthesis. By our solid-phase synthesis, chain elongation from the 3'- to the 5'-end, it is impossible to remove the unreacted 5'-hydroxyl oligomers. After repeating many cycles of the coupling reaction, the desired final product is not a major product if the unreacted oligomers are not masked at each cycle. Assuming 90% of the available 5'-hydroxyl groups on the solid-phase react with the 3'-phosphodiester component at each coupling (this is probably the maximum yield by our solid-phase

method), after six couplings the desired final product is only  $(0.90)^6 \times 100 = 53\%$  among other oligonucleotides. Thus, unmasking of the unreacted 5'-hydroxyl group caused some inconveniences. For example, the analysis of the dimethoxytrityl groups liberated from the resin after coupling reactions did not give exact information on the coupling yields and the incoming 3'-phosphodiester components were wasted to react with 5'-hydroxyl groups of undesired shorter oligonucleotides<sup>1</sup>. For the masking of the 5'-hydroxyl functions, two reagents were used, acetic anhydride and phenyl isocyanate. After every coupling reaction, the resin was treated with 10% solution of acetic anhydride or phenyl isocyanate in pyridine for 1 hour and then the dimethoxytrityl group was removed. Although the dimethoxytrityl group has some disadvantages for the protection of the 5'-hydroxyl group, as discussed above, this group makes it possible to immediately estimate the coupling yields without any special treatment of the resin.

#### Purification and Analysis of Polynucleotides

As described in the accompanying paper, the efficient separation of the final reaction mixture, including not only desired oligomers but also a series of shorter oligomers, was an essential requirement for a reliable synthesis. Permaphase AAX column chromatography, was quite satisfactory in the case of the dT(pT)<sub>18</sub> synthesis. The analysis of the product at each cycle, after removal of all the protecting groups, by high performance liquid chromatography (HPLC) on the Permaphase AAX column revealed that the synthesis of polynucleotides with defined sequences on the solid-phase was also more complicated than those of polythymidylic acid as observed in the solution method. Consequently, the final product isolated by the ion-exchanger was further analyzed and purified by  $\mu$ Bondapak C<sub>18</sub> reverse-phase chromatography<sup>8</sup>. The separation was achieved with linear acetonitrile gradient at 55°C at pH 7.0 (Figures 2 and 3). The separation of the reverse-phase column is very sensitive to the lipophilicity of the products and the small peaks having longer retention time than the desired major peaks were always observed. These peaks probably contained not completely deblocked polynucleotides that still have protecting groups for amino functions

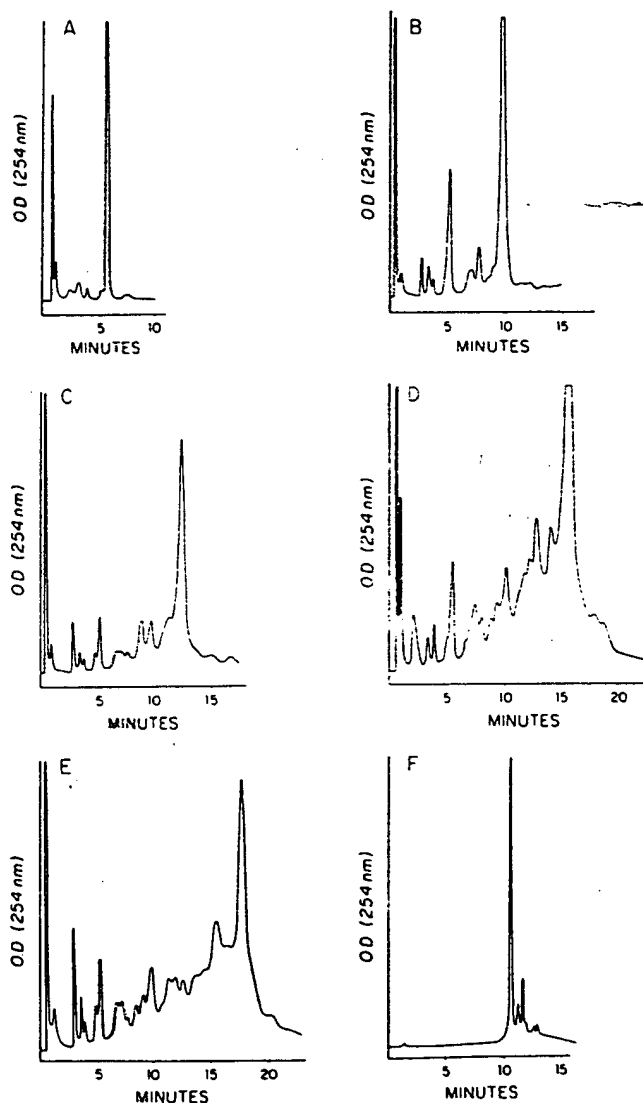


FIGURE 2. HPLC Analysis of the Synthesis of dTGCCGGCCACGATGCG.

After each coupling cycle, all the protecting groups were removed and the resultant oligonucleotides were fractionated on a Permaphase AAX column under the exact conditions previously reported. At A: the first cycle (tetramer, dTGCG); B: the second cycle (heptamer, dCGATGCG); C: the third cycle (decamer, dCCACGATGCG); D: the fourth cycle (tridecamer, dCGGCCACGATGCG); and E, the last cycle (hexadecamer, dTGCCGGCCACGATGCG). F: The last peak of E was further fractionated on  $\mu$ Bondapak C<sub>18</sub> after desalting under the conditions as described in the Experimental Section.

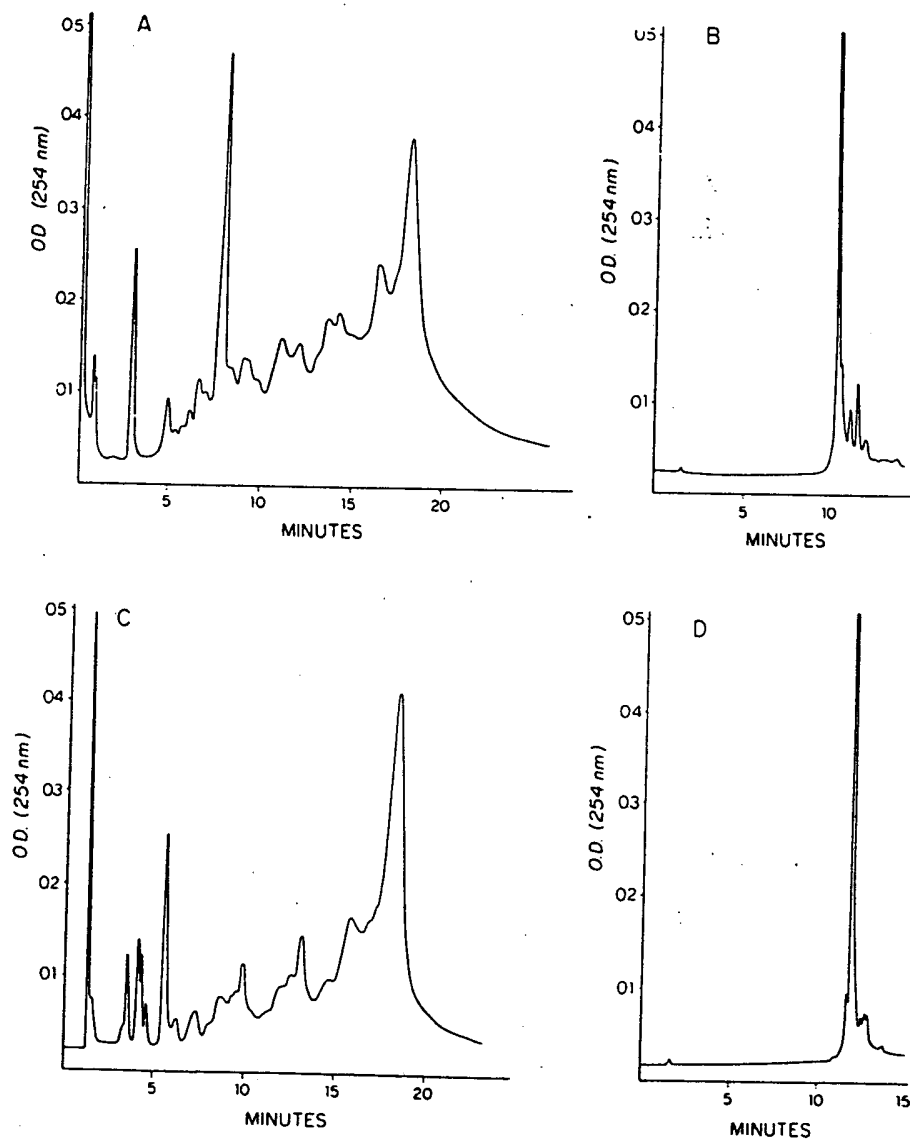


FIGURE 3. HPLC Analysis of Hexadecamers by Two Different Principles.

At A, the fractionation of the final cycle to synthesize dGTATCA CGAGGCCCTT on a Permaphase AAX column; B, further analysis of the last peak in A on  $\mu$ Bondapak C<sub>18</sub>. At C and D, the analysis of dCGACGAGCGTG ACACC on a Permaphase AAX and a  $\mu$ Bondapak C<sub>18</sub> column, respectively.



## Nucleic Acids Research

or phosphate groups. The analysis and purification of the desired product by these two different principles should be essential if the very pure polynucleotides are required. The desired product thus isolated was identified by the sequencing method<sup>15</sup>.

### General Procedure for the Synthesis

One cycle of the addition of an oligonucleotide unit to the solid-phase was essentially the same as described in the accompanying paper<sup>1</sup>, except for the additional step to mask the unreacted 5'-hydroxyl groups and a shorter time of the acidic treatment to remove the dimethoxytrityl group (see Table 1). A part of the resin at each cycle was removed and treated successively with aqueous ammonia ( $d = 0.88$ ) and 80% acetic acid and the reaction mixture of each coupling reaction was analyzed by high performance liquid chromatography on Permaphase AAX.

The typical HPLC profile of each cycle to synthesize the hexadecanucleotide, dTpGpCpCpGpGpCpCpApCpGpApTpGpCpG, was shown in Figure 2. The HPLC profiles of the other hexadecamers were shown in Figure 3. These polynucleotide sequences are being used as primers for the DNA sequencing developed by Sanger<sup>16</sup> and the results will be published elsewhere.

The above experiments demonstrated that the new solid-phase synthesis using oligonucleotide blocks as an additional unit at

TABLE 1.

STEP	SOLVENT or REAGENT	AMOUNT	SHAKING TIME (Minutes)	NUMBER OF OPERATIONS
1	2% BSA	10 ml	0.5	2
2	CHCl <sub>3</sub> -MeOH (7:3 v/v)	10 ml	1	2
3	Pyridine	10 ml	1	2
4	Dimer or Trimer in Pyridine	5 equivalent	co-evaporation	3
5	TPST in Pyridine	10 equivalent / 5 ml	180	
6	Pyridine	10 ml	1	2
7	10% (AcO) <sub>2</sub> O or PhNCO in Pyridine	10 ml	60	1
8	Pyridine	10 ml	1	2
9	CHCl <sub>3</sub> -MeOH (7:3 v/v)	10 ml	1	3

each cycle is very powerful for the rapid synthesis of polydeoxyribonucleotides. Furthermore, use of the rapid and efficient purification method by HPLC and the improved synthetic method for oligonucleotide blocks<sup>17</sup> have contributed significantly to increasing speed and accuracy of the synthesis. Although sequence-specific polydeoxyribonucleotides can be easily synthesized and isolated with a good yield by our method, there still remain some aspects to be investigated, including automation of all cycles by machine, depurination of N-benzoyl deoxyadenosine, and the length of polynucleotides to be synthesized on the solid-phase. These works are under continuing research.

## EXPERIMENTAL SECTION

### Material and Methods

Materials and methods not otherwise mentioned here have been described in previous papers.<sup>1,17</sup>

### Synthesis of Monosuccinates (IX)

Anhydrous 5'-O-dimethoxytrityl nucleoside (10 mmole) in pyridine (40 ml) was reacted with succinic anhydride (1.50 g, 15 mmole) in the presence of dimethylaminopyridine (1.83 g, 15 mmole). The reaction mixture was stirred at room temperature overnight and concentrated to a gum, which was taken up in  $\text{CHCl}_3$ . The  $\text{CHCl}_3$  solution was washed with 0.1 M  $\text{NaH}_2\text{PO}_4$  solution,  $\text{NaHCO}_3$  and water twice, dried ( $\text{Na}_2\text{SO}_4$ ) and evaporated. The product was chromatographically purified on silica gel (120 g) eluted with  $\text{CHCl}_3$ -MeOH solution (0-5% v/v). The isolated product was dissolved in a small amount of  $\text{CHCl}_3$ , and added dropwise to pentane (2 l). The precipitates were collected by filtration and dried. The yield was 83-90%.

### Preparation of Pentachlorophenyl Succinate (IV)

A mixture of the monosuccinate (IX, 2 mmole), pentachlorophenol (590 mg, 2.2 mmole) and dicyclohexyldiimide (620 mg, 3 mmole) in 15 ml of dimethylformamide (DMF) was stirred at room temperature for 20 hours. After filtration, the filtrate was evaporated and the residue was dissolved in benzene to remove the urea derivative. The product was precipitated by the dropwise addition of the benzene solution to pentane (400 ml). The

## Nucleic Acids Research

yield was 80-85%.

### Formation of Nucleoside-Polymer Linkage

The amino resin (5 g, 0.18 mmole/g) was reacted with the pentachloro ester (4.0 mmole) and triethylamine (0.44 g, 4.4 mmole) by shaking in 30 ml of DMF for 20 hours at room temperature. The mixture was filtered and the resin was washed with DMF and pyridine, and treated with phenylisocyanate (10% solution) in pyridine (35 ml) for 3 hours. The resin was filtered, washed with pyridine and MeOH, and dried. Both supports (V) had the same amount of nucleosides. The yield was: 0.15 mmole of deoxycytidine per 1 g of Vc and 0.15 mmole of deoxyguanosine per 1 g of Vd (see Table 2).

### Typical Synthesis of a Hexadecanucleotide, dTpGpCpCpGpGpCpCpApCpGpApTpGpCpG, using Trinucleotide Blocks

Each fully protected trinucleotide block (X, n=3, sequences of TGC-CGG-CCA-CGA-TGC) was synthesized as previously described<sup>17</sup> and decyanoethylated to give each trimer phosphodiester component. The nucleotide addition cycle (Table 1) was begun at Step 4 using a round-bottom flask, equipped with a sinister filter and two-way stopcock at the bottom, and manually operated system. A mixture of 300 mg of the hydroxyl resin (VId, 0.15 me g<sup>-1</sup> of N-isobutyryldeoxyguanosine), the first trimer phosphodiester component (TGC, 0.23 mmole) and pyridine (10 ml) was co-evaporated three times in the flask using a horizontal-type evaporator. To this mixture was added the coupling reagent, 2,4,6-triisopropylbenzenesulfonyl tetrazolide (TPST, 0.7 mmole),

TABLE 2.

BASE	NUCLEOSIDE ON RESIN*
Thymine (Va)	0.16 mmole/g
N-Benzoylcytosine (Vc)	0.15 mmole/g
N-isobutyrylguanine (Vd)	0.15 mmole/g

\* The amount of nucleosides linked on the resin was established by the quantitative analysis of both the dimethoxytrityl group and the nucleosides liberated from the resin. The yield of N-benzoyl adenine was not reported because of depurination.

and anhydrous pyridine (10 ml) and the mixture was shaken for 3 hours. The mixture was filtered by the application of dry air pressure through the top of the flask. The resin was treated with phenylisocyanate in pyridine (10% solution, 10 ml) under shaking for 1 hour to mask the unreacted 5'-hydroxyl groups. The reaction mixture was filtered and the resin was washed successively with pyridine (2 X 10 ml) and  $\text{CHCl}_3$ -MeOH (7:3 v/v, 3 X 10 ml). The dimethoxytrityl group was removed by treatment with a 2% benzenesulfonic acid solution (10 ml) in  $\text{CHCl}_3$ -MeOH (7:3 v/v) for 1 minute at room temperature. After washing the resin with  $\text{CHCl}_3$ -MeOH (7:3 v/v) two times, all the filtrates and washing solvents were pooled and the coupling yield was estimated by the spectroscopic analysis of the dimethoxytrityl function liberated from the resin. This coupling cycle was repeated five times and at the end of each cycle (Step 9 in Table 1) a sample of the resin (ca. 5 mg) was treated with concentrated  $\text{NH}_4\text{OH}$  (3 ml) for 20 hours at 50°C under shaking, and filtered. After evaporation of the filtrate, the residue was treated with 80% AcOH for 10 minutes at room temperature and evaporated. The HPLC profile of the resulting oligonucleotide at each cycle was reported in Figure 2 and the yield of each coupling cycle was 76, 90, 81, 85 and 95% for 1, 2, 3, 4 and 5 cycles, respectively.

The oligonucleotide chain was extended from the 3'-end to the 5'-end and Cycle 1 stands for the first coupling reaction between an oligonucleotide block (shown by a hyphen) and a nucleoside bound to the resin. Cycle 2 is a second coupling reaction of the resulting oligonucleotide chain bound on the resin with an incoming blocked nucleotide. For the synthesis of dGpTpApTpCpApCpGpApGpGpCpCpTpT, the exact same strategy described above was used. Each trimer block, GTA-TCA-CGA-GGC-CCT, was sequentially coupled to 300 mg of the resin (VIa, 0.16 meq  $\text{g}^{-1}$  of thymidine) and acetic anhydride was used for masking of the unreacted 5'-hydroxyl group. The yield of each coupling was 71, 76, 94, 78 and 90% for 1, 2, 3, 4 and 5 cycles, respectively. For the synthesis of dCpGpApCpGpApGpCpGpTpGpApCpApCpC, 300 mg of the starting resin (Vc, 0.15 meq  $\text{g}^{-1}$  of deoxycytidine) was coupled stepwise with each oligonucleotide block (CGA-CG-AG-CG-TGC-CAC). The yield of each step was 56, 98, 94, 87, 89 and

FIGURE 4a. Autoradiographs from Sequence Analysis of  
dG T A T C A C G A G G C C T T.

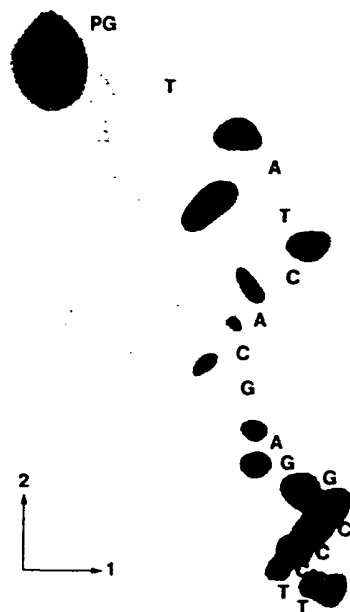


FIGURE 4b. Autoradiographs from  
Sequence Analysis of  
dC G A C G A G C G T G A C A C C.

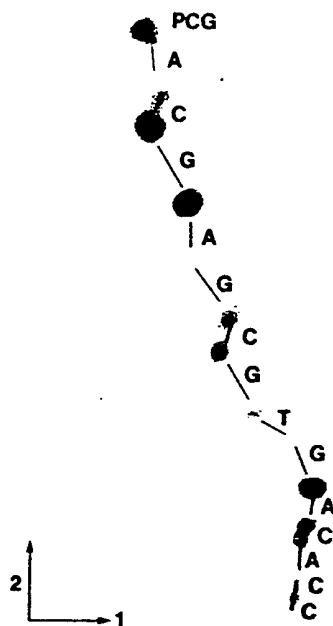
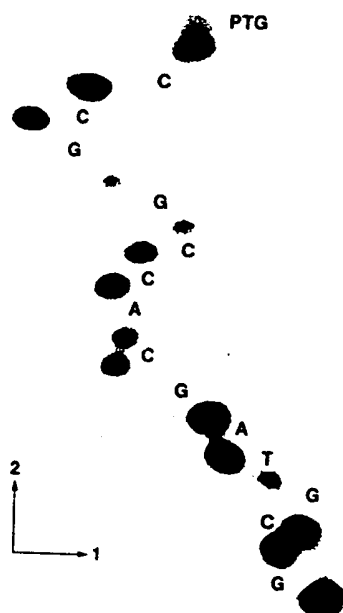


FIGURE 4c. Autoradiographs from  
Sequence Analysis of  
dT G C C G G C C A C G A T G C G.



81% for the sequential 6 cycle couplings. The HPLC profiles of these two hexadecanucleotides were shown in Figure 3.

#### HPLC Analysis and Purification of Oligonucleotides

Unprotected oligonucleotides were chromatographed on a Permaphase AAX column operated at 55°C under the exact conditions as reported.<sup>1</sup> The HPLC profiles were described in Figures 2 and 3. The major peak from the AAX column chromatography was de-salted by passing through a Sephadex G-25 column (1.5 X 50 cm) eluted with a 50 mM triethylammonium bicarbonate (TEAB) solution. After evaporation of the buffer, the sample was analyzed and further purified on  $\mu$ Bondapak C<sub>18</sub> reverse-phase column chromatography (Waters Associates). Columns (0.3 X 30 cm) were operated at 55°C using an SP3500 HPLC equipment with an oven (Spectra-Physics). Linear gradients generated from each of 0% CH<sub>3</sub>CN and 15% CH<sub>3</sub>CN at pH 7.0 (0.01 M ethylenediammonium acetate buffer); flow rates: 2 ml min<sup>-1</sup>; sweeping time: 15 minutes. The profiles of the hexadecamers were shown in Figures 2 and 3.

## Nucleic Acids Research

### Sequencing of Oligonucleotides

The oligonucleotides (0.01 A<sub>260</sub> unit) isolated from the reverse-phase column chromatography was treated with [ $\gamma$ -<sup>32</sup>P]ATP and T4 polynucleotide kinase under standard conditions and separated by chromatography on Sephadex G-50 (50 mM TEAB buffer). After evaporation of the buffer, partial treatment with snake venom phosphodiesterase, followed by separation as reported in two dimensions, showed a pattern of spots confirming the expected sequences (Figure 4).<sup>15</sup>

### ACKNOWLEDGEMENTS

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Rapid synthesis of oligodeoxyribonucleotides IV. Improved solid phase synthesis of oligodeoxyribonucleotides through phosphotriester intermediates

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ABSTRACT

A phosphotriester solid phase method on a polyamide support has been used to prepare oligodeoxyribonucleotides up to 12 units long. Compared to solid phase phosphodiester synthesis the new methodology is quicker, more flexible and gives 10-60-fold better overall yields.

INTRODUCTION

Previous papers in this series<sup>1-3</sup> have demonstrated the practical value of polydimethylacrylamide resins for the solid-phase synthesis of oligodeoxyribonucleotides. In these syntheses appropriate monomer units were coupled to the resin using a phosphodiester approach. Good coupling yields could be maintained provided that highly purified monomer units were used<sup>3</sup>. However, isolated yields of deprotected oligonucleotides decreased rapidly as a function of chain length owing to substantial accumulation of by-products. These we attribute mostly to the susceptibility of each phosphodiester bond to reaction with active phosphorylating agent during successive coupling steps<sup>4</sup>.

For some time we have felt that a phosphotriester approach applied to our polyamide resins might hold distinct advantages in terms of overall yields, speed of synthesis and length of oligonucleotide attainable, but only recently has sufficiently reliable solution methodology emerged<sup>5</sup>, particularly with regard to deprotection of phosphotriesters at the end of the synthesis<sup>6</sup>. Although a phosphotriester approach on solid phase has been attempted several times in the past<sup>7</sup>, only the very recently described synthesis of oligothymidylates on a polyacryloylmorpholide resin has been at all encouraging<sup>8</sup>. We now describe the solid phase synthesis of the octanucleotides, d(T-C-T-G-G-T-T-T), d(C-C-T-C-C-T-G-C), d(T-T-C-C-C-A-C-C) and d(C-T-C-C-C-A-C-C) and the dodecanucleotides, d(C-T-C-C-C-A-C-C-A-T-T-T) and d(T-T-C-C-C-A-C-C-A-T-T-T), obtained in good overall isolated yields using a phosphotriester approach.

## Nucleic Acids Research

### DISCUSSION

The Polymeric Support. The principles behind the choice of polydimethylacrylamide resins have been discussed at length<sup>1,9</sup>. Recently we introduced a new functional monomer, acryloylsarcosine methyl ester, which when copolymerised with dimethylacrylamide and ethylene bisacrylamide gave beaded resins in easily handlable form<sup>10</sup>. In peptide synthesis on polydimethylacrylamide resins it has been standard practice to incorporate a reference amino acid on the resin before starting chain assembly<sup>11</sup>. Yields may then be estimated by measurement of amino acid content of resin-bound peptides normalised to the value of the reference, hence eliminating errors associated with increase in resin weight. In oligonucleotide synthesis we now find that use of a reference amino acid allows easy measurement of overall yields of oligonucleotides. In addition functional groups (such as 4,4-dimethoxytrityl) can be assayed with respect to the amino acid content of the resin to give a useful guide to the efficiency of coupling reactions.

The new resin is functionalised by treatment with ethylene diamine at room temperature and the resultant amino groups then reacted with the symmetrical anhydride of t-butyloxycarbonylglycine in DMF (Fig. 1). After washing the resin (gly 0.28-0.35 mmole g<sup>-1</sup>) may be dried and stored.

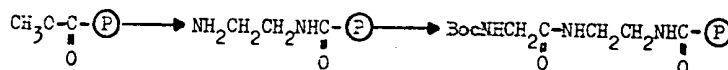


Fig. 1.

The Resin-Polymer Linkage. In our phosphodiester approach a reversible linkage was obtained by reaction of a suitably protected 2'-deoxynucleoside-5'-phosphate with a resin-bound β-hydroxyethylthiophenol derivative<sup>1</sup>. The resultant phosphodiester could be cleaved by oxidation to the corresponding sulphone followed by base-catalysed elimination. Whereas this linkage was stable indefinitely in the presence of pyridine an analogous 3'-phosphotriester linkage formed by coupling of the hydroxy-resin with (MeO)<sub>2</sub>TrdT-(ClPh) was ca 30% cleaved in one week at room temperature in pyridine and was therefore judged unsuitable.

Among other base-labile linkages nucleoside derivatives have been attached to succinylated polystyrene resins as 5'-O-esters<sup>12</sup>. In line with our proposed chain extension procedure we wished to attach the first nucleoside residue via its 3' position<sup>13</sup>. To avoid the possibility of unsubstituted and potentially deleterious carboxylic acids<sup>3</sup> remaining on the resin appropriate 2'-deoxynucleoside derivatives were themselves succinylated and then coupled

to the resin. Accordingly  $(\text{MeO})_2\text{TrdT}$ ,  $(\text{MeO})_2\text{TrdbzA}$ ,  $(\text{MeO})_2\text{TrdbzC}$  and  $(\text{MeO})_2\text{TrdibG}$  were each treated with succinic anhydride in the presence of 4-dimethylaminopyridine in DMF for 20 h at room temperature and the corresponding 3'-O-succinates isolated as their pyridinium salts following preparative layer chromatography. In the presence of dicyclohexylcarbodiimide in dichloromethane the succinates readily formed their respective symmetrical anhydrides which, after removal of dicyclohexylurea, were reacted in DMF with amino groups on the resin liberated by acidic cleavage of t-butyloxycarbonyl protecting groups and neutralisation (Fig. 2). Completion of reaction was

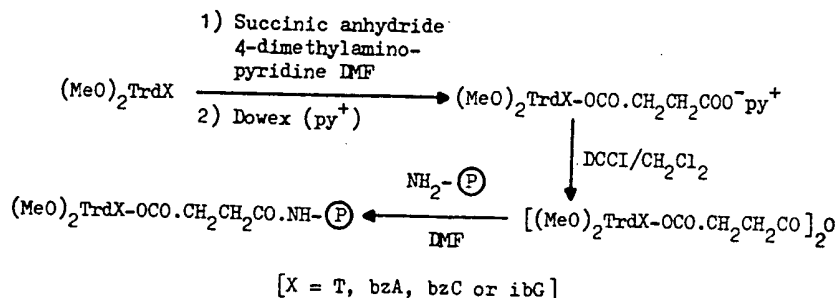


Fig. 2.

judged by the ninhydrin test<sup>14</sup>. An advantage of this procedure is that the succinates are the only by-products remaining in solution and can be recovered for re-use. The linkage is stable in the presence of pyridine and under acidic conditions [5% benzene sulphonic acid in chloroform/methanol (7:3)] but is rapidly cleaved by aqueous sodium hydroxide or ammonia treatment.

**Oligonucleotide Assembly.** For the most part the phosphotriester approach as modified by Stawinski *et al.*<sup>5</sup> has been used. Basic units are  $(\text{MeO})_2\text{TrdT}-(\text{ClPh})$ ,  $(\text{MeO})_2\text{TrdbzA}-(\text{ClPh})$ ,  $(\text{MeO})_2\text{TrdbzC}-(\text{ClPh})$  and  $(\text{MeO})_2\text{TrdibG}-(\text{ClPh})$  and corresponding di- and trinucleotide blocks<sup>15</sup>, which are coupled to the resin using triisopropylbenzenesulphonyltetrazole (Fig. 3). These reactions alter-

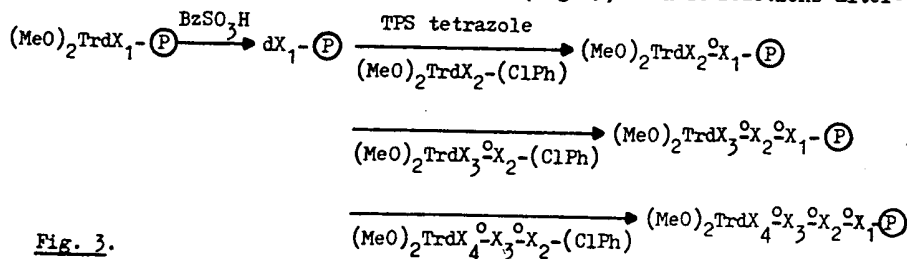


Fig. 3.

## Nucleic Acids Research

nate with terminal 5'-deprotection using 5% benzene sulphonic acid in chloroform/methanol (7:3)<sup>16</sup>. Each complete synthetic cycle can be carried out in 5-6 h using a bench-top manual solvent delivery system<sup>1</sup> or a modified Beckman 990B Solid Phase Peptide Synthesiser<sup>2,17</sup> using essentially similar operations (Table 1, Experimental Section). For oligonucleotides containing N-benzoyl adenine the minimum of acidic treatment is vital to prevent depurination. In the syntheses described below two 30s treatments followed by rapid resin washing were sufficient for complete deprotection without concomitant depurination, but the precise limits of this procedure in the case of adenine-rich oligonucleotides have yet to be determined. In contrast to the phosphodiester approach resin drying with phenyl isocyanate<sup>1,2</sup> is not necessary during synthesis, although a precautionary 30m treatment is given at the start of the synthesis.

**Oligonucleotide Cleavage and Isolation.** Aqueous ammonia has most commonly been used for cleavage of aryl groups from internucleotide linkages<sup>8,18</sup>. This procedure also liberates oligonucleotides from the resin and removes all base-protecting groups. Dimethoxytrityl groups may then be removed with acetic acid (Fig. 4, Procedure A). However, the use of ammonia for removal

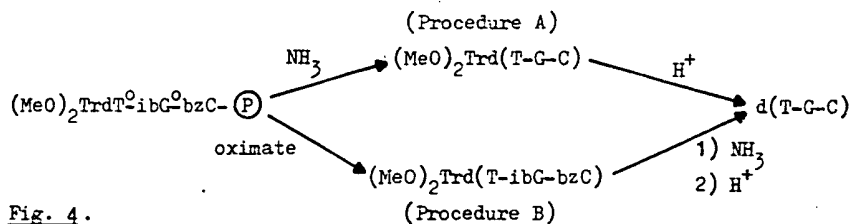


Fig. 4.

of aroyl groups has been shown to cause considerable chain degradation<sup>6</sup>. We have confirmed these observations and shown that alternative use of 0.3 M tetramethylguanidinium p-nitrobenzaldoximate<sup>6</sup> leads to higher yields of the desired oligonucleotide. The reagent also cleaves the oligonucleotide-resin linkage. Further deprotection of the detached oligonucleotide with ammonia and acetic acid proceeds as before (Procedure B).

The completely deprotected oligonucleotide is purified by ion-exchange hplc<sup>2,3</sup>. This is particularly advantageous in that in contrast to reversed phase hplc the desired oligonucleotide is always eluted later than any truncated or failure sequence and can be readily identified. After desalting the product is then assayed for purity by reversed phase hplc on  $\mu$ -Bondapak C18<sup>10,19</sup> and by sequencing of <sup>32</sup>P-labelled samples.

**Synthesis of Oligonucleotides.** Three alternative strategies were investi-

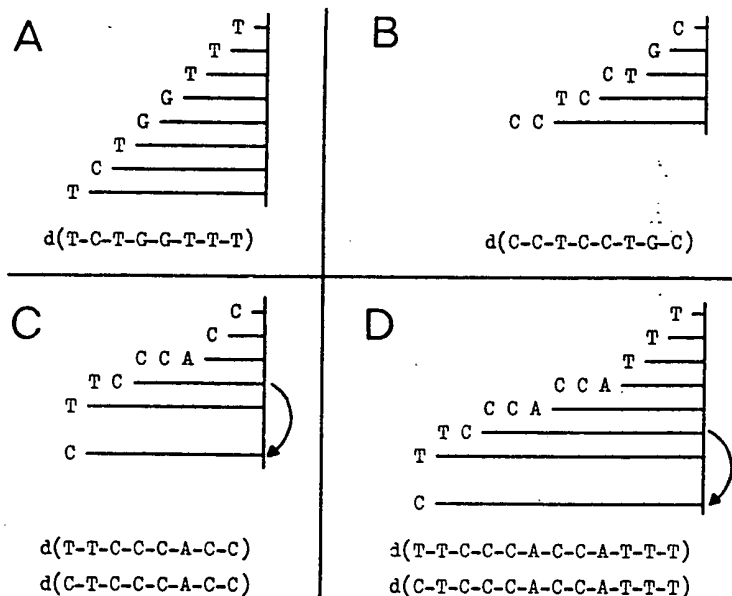


Fig. 5. Schematic plan for assembly of oligonucleotides.

gated for the synthesis of octanucleotides (Fig. 5, A-C). The octanucleotide d(T-C-T-G-G-T-T-T) was assembled purely by the addition of monomer units, the octanucleotide d(C-C-T-C-C-T-G-C) by successive addition of a monomer and three dimers, the octanucleotides d(T-T-C-C-C-A-C-C) and d(C-T-C-C-C-A-C-C) by addition of monomers, one dimer and one trimer. A nearly four-fold increase in overall yield of the first octanucleotide (Table 2) was obtained by use of deprotection procedure B rather than A. The improvement can also be seen by comparison of the chromatographic patterns obtained (Fig. 6) where both the product peak and peaks corresponding to shorter chain intermediates are substantially larger. The purity of the octanucleotide as judged by  $\mu$ -Bondapak C18 hplc<sup>20</sup> was also increased.

In the synthesis of the other three octanucleotides (Table 2) deprotection procedure B was used throughout and the chromatographic patterns (Figs. 7-9) all show clear product peaks (yields 4.1-7.8%).

In the synthesis of the dodecanucleotides d(T-T-C-C-C-A-C-C-A-T-T-T) and d(C-T-C-C-C-A-C-C-A-T-T-T) the assembly strategy involved use of both monomers and preformed blocks (Fig. 5, D). Whereas deprotection procedure A was adequate for shorter chain intermediates (Table 3) overall yields of dodecamers dropped to 1.7 and 1.2% respectively. In the latter case use of

# Nucleic Acids Research

TABLE 2

Sequence	Resin sample (mg)	Glycine (μmole)	Cleavage method	Product isolated		Overall yield %	Purity % (Bondapak C18)
				(A <sub>260</sub> )	(μmole)		
d(T-C-T-G-G-T-T-T)	44.53	7.19	A	8.0	0.107 <sup>1</sup>	1.8	87
d(T-C-T-G-G-T-T-T)	66.20	10.43	B	36.3	0.486 <sup>2</sup>	6.8	93
d(C-C-T-C-C-T-G-C)	16.59	2.58	B	7.0	0.106	4.1	90
d(T-T-C-C-C-A-C-C)	9.76	1.92	B	9.9	0.142	7.4	94
d(C-T-C-C-C-A-C-C)	26.75	5.19	B	27.6	0.406	7.8	95

Note: <sup>1</sup> Only 85% of cleavage product purified; <sup>2</sup> Only 69% of cleavage product purified

deprotection procedure B doubled the yield (2.5%) and the product purity was increased. Chromatographic patterns of the two dodecanucleotides (Figs. 10 and 11) deprotected by different routes also reflect this improvement.

Certain direct comparisons can be made between the present phosphotriester syntheses and our original phosphodiester approach<sup>1-3</sup>. Individual coupling yields assessed by ratio of peak areas on hplc chromatograms are apparently similar (80-90% for monomer addition, 60-80% for blocks) but give a misleading impression. Instead the overall isolated yield of octanucleotides (4.1-

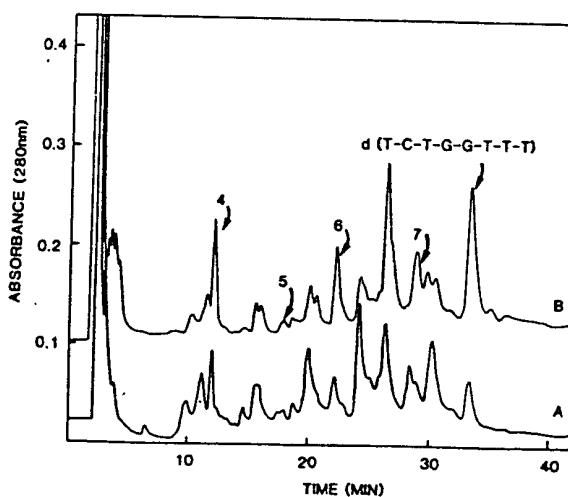


Fig. 6. Chromatographic patterns of the octanucleotide d(T-C-T-G-G-T-T-T) following deprotection procedures A and B.

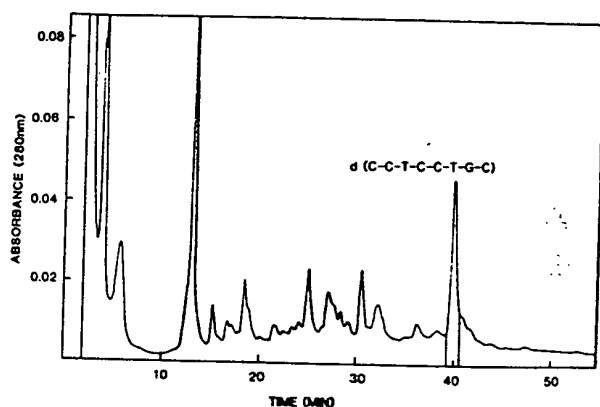


Fig. 7. Chromatographic pattern of the octanucleotide d(C-C-T-C-C-T-G-C)

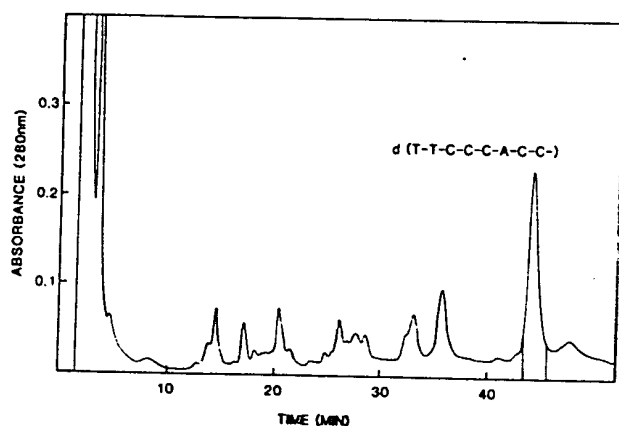


Fig. 8. Chromatographic pattern of the octanucleotide d(T-T-C-C-C-A-C-C-)

7.8%) and dodecanucleotides (1.7-2.5%) should be compared with approximate values of 0.5%<sup>2</sup> and 0.04%<sup>3</sup> obtained respectively for an octanucleotide and a dodecanucleotide synthesised by the phosphodiester approach. This 10-60-fold increase in overall yield is attributable to a substantial reduction in formation of resin-bound by-products and to improved efficiency of cleavage of oligonucleotides from the resin. Chain assembly is faster and the procedure more flexible; equally good yields are obtained in routes involving both monomer and block addition. Moreover, oligonucleotides of satisfactory purity can be isolated by a single chromatographic step<sup>21</sup>. We intend to extend this

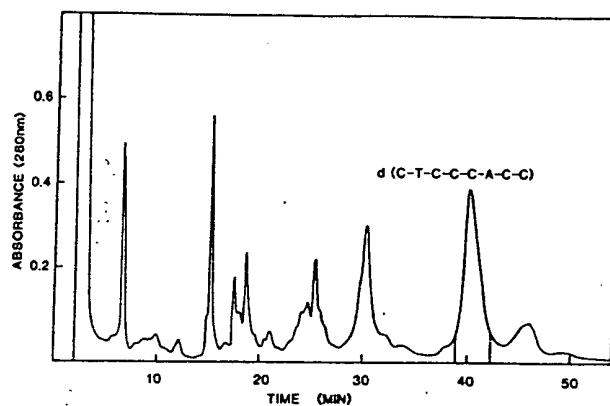


Fig. 9. Chromatographic pattern of the octanucleotide d(C-T-C-C-C-A-C-C)

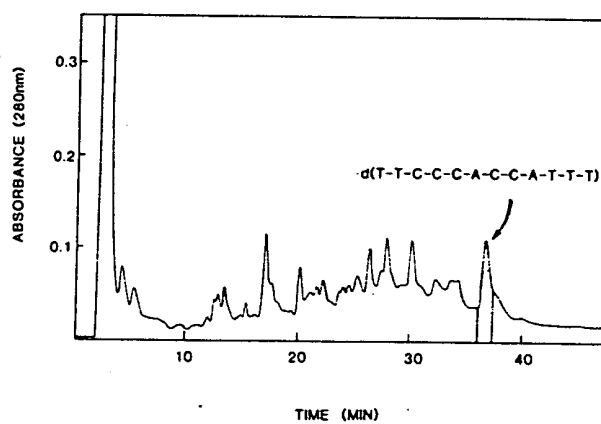


Fig. 10. Chromatographic pattern of the dodecanucleotide d(T-T-C-C-C-A-C-C-A-T-T-T); deprotection procedure A

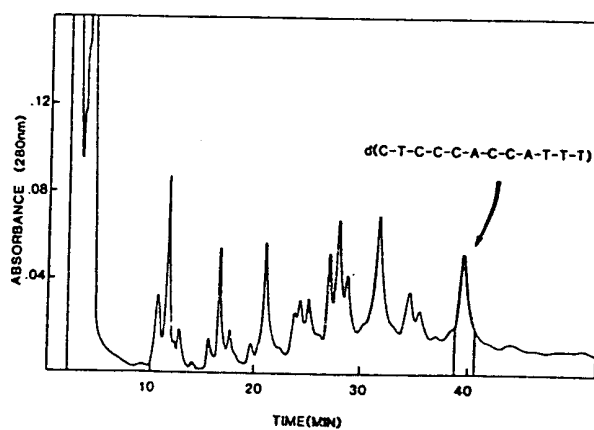


Fig. 11. Chromatographic pattern of the dodecanucleotide d(C-T-C-C-C-A-C-C-A-T-T-T); deprotection procedure B



TABLE 3

Sequence	Resin sample (mg)	Glycine (mmole)	Cleavage method	Product isolated		Overall yield %	Purity % (± Bondapak C18)
				(A <sub>260</sub> )	(mmole)		
d(C-C-A-T-T-T)	13.57	3.148	A	23.8	0.422	13.4	93
d(C-C-A-C-C-A-T-T-T)	7.45	1.687	A	9.0	0.104	6.2	93
d(T-C-C-C-A-C-C-A-T-T-T)	24.14	5.180	A	9.6	0.094	1.8	86
d(T-T-C-C-C-A-C-C-A-T-T-T)	29.92	5.592	A	10.8	0.097	1.7	75
d(C-T-C-C-C-A-C-C-A-T-T-T)	18.20	3.452	A	4.6	0.042	1.2	74
d(C-T-C-C-C-A-C-C-A-T-T-T)	7.47	1.409	B	3.8	0.035	2.5	88

approach to the synthesis of both adenine-rich and longer oligonucleotides and believe that this rapid methodology will find wide application in the preparation of biologically useful oligonucleotides.

#### EXPERIMENTAL SECTION

Unless otherwise mentioned materials and methods are as previously described<sup>1-3</sup>. 4,4-Dimethoxytrityl chloride and p-chlorophenylphosphorodichloridate were obtained from Lancaster Synthesis, 1,2,4-triazole, 1-H tetrazole, 3-hydroxypropionitrile and 4-dimethylaminopyridine from Aldrich, succinic anhydride from BDH, benzenesulphonic acid, electronic grade, from Eastman, thymidine from Sigma, deoxyadenosine from Calbiochem, deoxyguanosine and deoxycytidine hydrochloride from Leon Laboratories, St. Louis. Koch-Light were suppliers of 1,1,3,3,-tetramethylguanidine and p-nitrobenzyl alcohol from which p-nitrobenzaldehyde<sup>22</sup> and then p-nitrobenzaldoxime<sup>23</sup> were prepared. Fully protected nucleoside and oligonucleotide phosphotriester building blocks were prepared essentially as previously described<sup>5,24</sup>. Silica gel column chromatography was carried out on a Waters prep. LC/system 500 using methanol/dichloromethane solvent mixtures or on a Merck Lobar column, size C, using methanol/chloroform-0.5% pyridine eluants. Removal of cyanoethyl protecting groups followed the procedure of Sood and Narang<sup>25</sup> except that the phosphodiester products were freed from cyanoethanol by partition between chloroform and saturated brine. The organic phase was evaporated and product precipitated with diethyl ether/n-pentane (3:2). Hplc was carried out using an Altex modular system consisting of two 110 pumps, 420 system controller and 210 deluxe injection valve. Column effluents were monitored by a Cecil 212A UV photometer linked to a Tekman TE 220 recorder. Partisil 10SAX columns (PXS-analytical or M9-preparative, Whatman) were eluted with gradients of potassium phosphate (pH 6.5)/5% ethanol from 1 mM (pump A) to

## Nucleic Acids Research

0.2 M (pump B) at ambient temperature. These columns appear to have limited lifetime (30-50 injections) due to voiding caused by dissolving of the packing material. Small voids can be rectified by filling the space with glass beads, but we are informed by the manufacturer that a small precolumn of silica before the injector reduces this problem by presaturating the eluant. Eluant pH should not be dropped as this results in considerable loss of resolution and peak delay.  $\mu$ -Bondapak C18 (Waters) columns were eluted with 0.1 M ammonium acetate/acetonitrile solvent mixtures.

All resin treatments were carried out in an all-glass reaction vessel as previously described using a bench-top valve system<sup>1,2</sup> or a modified Beckman 990B solid phase peptide synthesiser<sup>2,16</sup>. Resin prefunctionalisation was carried out in a 200 ml capacity vessel using 140 ml solvent per wash. Resin (5 g) was treated with anhydrous ethylene diamine (Fluka, 170 ml) for 16 h and the resin washed with DMF until the eluate gave no blue colour in the ninhydrin test<sup>14</sup> (10-15 washes). The resin was then treated with (1) 3 x 10% diisopropylethylamine/DMF, 5 min, (2) 5 x DMF, 2 min, (3) 1 x 4 equivalents of the symmetrical anhydride of Boc gly, 90 min [Boc gly (12 mmole) and DCCI (6 mmole) in the minimum volume of dichloromethane were stirred for 15 min at room temperature, the mixture filtered, evaporated to dryness and dissolved in DMF (100 ml) just prior to use], (4) 5 x DMF, 2 min, (5) 1 x 10% diisopropylethylamine/DMF, 5 min, (6) 3 x DMF, 2 min, (7) 5 x dichloromethane, 5 min, (8) 5 x dioxan, 5 min, (9) 5 x diethyl ether, 5 min. The resin was dried in vacuo and assayed for glycine<sup>26</sup> using a Beckman 119C amino acid analyser with  $\alpha$ -amino- $\beta$ -guanidinopropionic acid as internal standard.

General Procedure for Preparation of Pyridinium 5'-O-dimethoxytrityl-2'-deoxynucleoside-3'-O-succinates. The 5'-O-dimethoxytrityl deoxynucleoside (dT,dbzA,dbzC or dibG) (3.35 mmole) was dissolved in DMF (15 ml) and succinic anhydride (6.7 mmole) and 4-dimethylaminopyridine (6.7 mmole) added. After 20 h at room temperature the mixture was evaporated to dryness and dissolved in pyridine/water (2:3). The solution was passed slowly through a column of Dowex 50-X8 (pyridinium). The eluate and column washings were evaporated to dryness and co-evaporated with pyridine to a foam, which was dissolved in chloroform/0.1% pyridine and applied to 10 preparative silica plates (20 x 2 mm, Merck 5717). The plates were eluted with chloroform/ethanol/pyridine (100:10:0.1) and the major band (UV and trityl positive) in each case scraped off and together eluted batchwise with chloroform/ethanol/pyridine (80:20:0.5). The eluate was evaporated to dryness and product precipitated with diethyl ether/pentane (3:2). Tlc analysis of the product on silica in chloro-

form/ethanol/pyridine (90:10:0.1) is essential to ensure the absence of pyridinium succinate ( $R_f$  0.1-0.2) which will seriously interfere with coupling of the nucleoside derivative to the resin. Yields 30-60% - as partial pyridinium salts:-

5'-O-Dimethoxytrityl-2'-deoxythymidine-3'-O-succinate;  $\lambda$  max (ethanol) 234, 266,  $\lambda$  min 254 nm; ir  $1735\text{ cm}^{-1}$  (C=O stretch succinate); nmr  $\delta$  9.97 (s, 1H, NH-3) 7.7-6.7 (m, 14H, Ar+H-6) 6.38 (tr, 1H, H-1') 5.45 (m, 1H, H-3') 4.16 (m, 1H, H-4') 3.80 (s, 6H, CH<sub>3</sub>O-) 3.45 (m, 2H, H-2') 2.65 (s+m, 6H, H-5'+ succinate) 1.38 (s, 3H, CH<sub>3</sub>-5).

5'-O-Dimethoxytrityl-N<sup>6</sup>-benzoyl-2'-deoxyadenosine-3'-O-succinate;  $\lambda$  max 233, 278 (br),  $\lambda$  min 256; ir 1740; nmr  $\delta$  8.67 (s, 1H, H-2) 8.22 (s, 1H, H-8) 8.1-6.6 (m, 18H, Ar) 6.45 (tr, 1H, H-1') 5.50 (m, 1H, H-3') 4.28 (m, 1H, H-4') 3.76 (s, 6H, CH<sub>3</sub>O-) 3.40 (m, 2H, H-2') 2.64 (s+m, 6H, H-5'+ succinate).

5'-O-Dimethoxytrityl-N<sup>4</sup>-benzoyl-2'-deoxycytidine-3'-O-succinate;  $\lambda$  max 236, 258, 284 (sh), 304,  $\lambda$  min 250, 290; ir 1740; nmr  $\delta$  8.16 (d, 1H, H-6) 8.04-6.70 (m, 19H, H-5+Ar) 6.26 (tr+s, 2H, H-1+NH-4) 5.41 (m, 1H, H-3') 4.24 (m, 1H, H-4') 3.80 (s, 6H, CH<sub>3</sub>O-) 3.43 (m, 2H, H-2') 2.68 (s+m, 6H, H-5'+ succinate).

5'-O-Dimethoxytrityl-N<sup>2</sup>-isobutyryl-2'-deoxyguanosine-3'-O-succinate;  $\lambda$  max 236, 252 (sh), 260 (sh), 274, 280,  $\lambda$  min 270; ir 1735; nmr  $\delta$  9.65 (s, 1H, NH-1) 7.76 (s, 1H, H-8) 7.4-6.6 (m, 13H, Ar) 5.96 (tr, 1H, H-1') 5.44 (m+s, 2H, H-3'+NH-2) 4.22 (m, 1H, H-4') 3.77 (s, 6H, CH<sub>3</sub>O-) 3.30 (m, 2H, H-2') 2.69 (s+m, 7H, H-5'+succinate+CO-CH) 1.10 (tr or d.d., 6H, CH<sub>3</sub>-C).

(N.B. All UV spectra were identical to the corresponding nucleoside starting materials.)

Attachment of Deoxynucleoside-3'-O-succinate derivatives to the Resin. A resin batch containing  $0.31\text{ mmole g}^{-1}$  of glycine was used in all experiments. Ca. 10 ml of solvent was used per wash per 0.35 g resin. Resin was swollen in DMF and treated with (1) 5 x t-amyl alcohol, 2 min, (2) 5 x acetic acid, 2 min, (3) 2 x 1.5 M hydrogen chloride in acetic acid, 5 + 25 min, (4) 5 x acetic acid, 2 min, (5) 10 x DMF, 2 min, (6) 3 x 10% diisopropylethylamine/DMF, 2 min, (7) 5 x DMF, 2 min, (8) 1 x 2 equivalents of the symmetrical anhydride of the succinate derivative (ir  $1822\text{ cm}^{-1}$ , C=O stretch; formed from 4 equivalents of the succinate by the same procedure as for Boc gly), 120 min, (9) 5 x DMF, 2 min. A sample of resin was washed with 5 x dichloromethane, 2 min, and then 5 x diethylether, 2 min, and dried in vacuo. Three or four resin particles were assayed by the ninhydrin test<sup>14</sup> whilst 1-2 mg samples were analysed for glycine content and trityl incorporation respectively (determined spectrophotometrically in 5 ml of 60% perchloric acid/ethanol

## Nucleic Acids Research

[3:2],  $\epsilon_{500} = 71,700$ ). The nucleoside derivative should be quantitatively incorporated (ca. 0.25 mmole  $g^{-1}$  of final resin, trityl/gly 1.0).

**Oligonucleotide Assembly.** The resin is washed with (1) 5 x pyridine, 2 min, (2) 1 x 10% phenylisocyanate/pyridine, 30 min, (3) 5 x pyridine, 2 min, followed by the appropriate number of synthetic coupling cycles (Table 1).

**Deprotection and Cleavage from the Resin. Procedure A.** The resin sample is treated with concentrated ammonia (1 ml per 10 mg resin) for 5 h at 50°, the liquid decanted and resin washed with ethanol/water (1:1) (3 x 1 ml). The decantate and washings are evaporated to dryness and the residue treated with acetic acid/water (4:1; 1 ml) for 30 min at room temperature. After

TABLE 1

Step	Reagent or solvent	Time of shaking (min)		No. of operations	
		Adenine absent	Adenine present	Adenine absent	Adenine present
1	CHCl <sub>3</sub> /MeOH (7:3)		2	10	
2	CHCl <sub>3</sub> /MeOH (7:3)		5	3 <sup>1</sup>	
3	5% benzene sulphonic acid in CHCl <sub>3</sub> /MeOH (7:3)	1	0.5	3	1 <sup>2</sup>
4	CHCl <sub>3</sub> /MeOH (7:3)	2	0.1	5	5 <sup>3</sup>
5	DMF		2	5	
6	Pyridine		2	10	
7	Coupling mixture		180	1 <sup>4</sup>	
8	Pyridine		2	5 <sup>5</sup>	

- <sup>1</sup> The extended washing procedure is necessary to ensure reproducible removal of pyridine, which would otherwise neutralise the acid subsequently added.
- <sup>2</sup> An orange colour is immediately liberated into solution on addition of the acid.
- <sup>3</sup> For oligonucleotides containing adenine steps 3 and 4 are repeated (usually once) until no further orange colour is seen. A sample of dried resin can also be assayed for trityl content.
- <sup>4</sup> The nucleoside or oligonucleotide 3'-phosphate (5 equivs) is dried by co-evaporation three times with anhydrous pyridine and in a dry box triisopropylbenzene sulphonyltetrazole (8.75-10 equivs) added. After 5-10 min the mixture is introduced to the resin (1 equiv as glycine) via the Teflon feed lines<sup>1,2</sup> or by Pasteur pipette.
- <sup>5</sup> 10 Washes are usually given in automatic operation. The programme also contains an instruction to hold until the operator is ready to begin again at step 1.

evaporation the product is dissolved in ethanol/water (1:1) ready for hplc.

**Procedure B.** The resin sample is shaken with 0.3 M tetramethylguanidinium p-nitrobenzaldoximate in dioxan/water (1:1) (1 ml per 10 mg resin) for 15-18 h, the liquid decanted and resin washed with dioxan/water (1:1) (3 x 1 ml). The decantate and washings are carefully neutralised with acetic acid, washed with chloroform (1 ml) and the aqueous phase evaporated to dryness. The residue is treated with concentrated ammonia (1 ml) at 50° for 5 h, evaporated to dryness and treated with acetic acid/water (4:1; 1 ml) for 30 min. After evaporation the product is dissolved in ethanol/water (1:1) ready for hplc.

**The Octanucleotide d(T-C-T-G-G-T-T-T).** Resin (0.354 g, 0.109 mmole glycine) was derivatised as described above to obtain the 5'-O-dimethoxytrityl-thymidine-3'-O-succinamido resin (trityl 0.252 mmole g<sup>-1</sup>; trityl/gly 1.07). Seven cycles of nucleotide addition were carried out using the Beckman synthesiser and appropriate monomer units only. Resin samples at each stage gave trityl/gly values of 0.93, 0.91, 0.78, 0.82, 0.72, 1.11 and 0.95 respectively. A sample of final resin (44.53 mg, 7.19 µmole Gly) was treated according to cleavage procedure A. 85% of the product (λ max 269 nm, 311 A<sub>260</sub> units) was chromatographed on Partisil 10SAX (Fig. 6A, 5 injections, elution 6 min 2% buffer B, 20 min 2-25% B, 25 min 25-40% B) to give 8.0 A<sub>260</sub> units in the product peak (0.107 µmole, overall yield 1.75%). The material was desalted on Biogel P2 (λ max 266 nm, 260/280 1.408, calc. 1.351). µ-Bondapak C18 hplc assay showed 87% purity<sup>20</sup>. Another sample of final resin was treated according to cleavage procedure B. 69% of the product was chromatographed on Partisil 10SAX (Fig. 6B, 5 injections) to give 36.3 A<sub>260</sub> units (0.486 µmole, overall yield 6.75%) in the product peak. The material was desalted on Biogel P2 (λ max 265 nm, 260/280 1.363, calc. 1.351). µ-Bondapak C18 hplc assay showed 93% purity. The sequence was confirmed by standard analysis of a <sup>32</sup>P-labelled sample.

**The Octanucleotide d(C-C-T-C-C-T-G-C).** Resin (0.139 g, 0.043 mmole glycine) was derivatised as described above to obtain the 5'-O-dimethoxytrityl-N<sup>4</sup>-benzoyl-2'-deoxycytidine-3'-O-succinamido resin (trityl 0.270 mmole g<sup>-1</sup>, trityl/gly 1.27). Using the manual solvent delivery system one cycle of assembly was carried out with (MeO)<sub>2</sub>TrdibC-(ClPh) in the coupling step followed by three cycles using in order the dinucleotide blocks (MeO)<sub>2</sub>TrdbzC<sup>0</sup>-(ClPh), (MeO)<sub>2</sub>TrdT<sup>0</sup>bzC-(ClPh) and (MeO)<sub>2</sub>TrdbzC<sup>0</sup>bzC-(ClPh). Trityl/gly values of resin samples at each stage were 1.35, 1.02, 0.95 and 0.54 respectively. A sample of final resin (16.59 mg, 2.578 µmole Gly) was treated

## Nucleic Acids Research

according to cleavage procedure B and the product chromatographed on Partisil 10SAX (Fig. 7, 3 injections, elution 6 min 8% B, 45 min 8-70% B). The material in the product peak was desalted on Biogel P2 to give 6.97  $A_{260}$  units (0.106  $\mu$ mole, overall yield 4.1%,  $\lambda$  max 270 nm, 260/280 1.200, calc. 1.148).  $\mu$ -Bondapak C18 hplc assay showed 90% purity. The sequence was confirmed by standard analysis of a  $^{32}$ P-labelled sample.

The Octanucleotides d(T-T-C-C-C-A-C-C) and d(C-T-C-C-C-A-C-C). Resin (0.154 g, 0.0476 mmole Glycine) was derivatised as described above to obtain the 5'-O-dimethoxytrityl-N<sup>4</sup>-benzoyl-2'-deoxycytidine-3'-O-succinamido resin (trityl 0.258 mmole g<sup>-1</sup>, trityl/gly 1.05). Using the manual solvent delivery system one cycle of assembly was carried out with (MeO)<sub>2</sub>TrdbzC-(ClPh), one with (MeO)<sub>2</sub>TrdbzC<sup>O</sup>-bzC<sup>O</sup>-bzA-(ClPh) and one cycle with the dinucleotide block (MeO)<sub>2</sub>TrdT<sup>O</sup>-bzC-(ClPh). Trityl/gly values at each stage were 0.92, 0.64 and 0.61 respectively. The resin was divided into two approximately equal portions. With each portion one cycle of assembly was carried out using (MeO)<sub>2</sub>TrdT-(ClPh) in the coupling step in one case and (MeO)<sub>2</sub>TrdbzC-(ClPh) in the other. A sample of the former final resin (9.765 mg, 1.925  $\mu$ mole Gly) was treated according to cleavage procedure B and the product chromatographed on Partisil 10SAX (Fig. 8, 2 injections, elution 6 min 5% B, 18 min 5-35% B, 27 min 35-45% B). The material in the product peak was desalted to give 9.9  $A_{260}$  units (0.142  $\mu$ mole, overall yield 7.4%,  $\lambda$  max 268 nm, 260/280 1.321 calc. 1.349).  $\mu$ -Bondapak C18 hplc assay showed 94% purity. A sample of the latter final resin was treated according to cleavage procedure B and the product chromatographed on Partisil 10SAX (Fig. 9, 2 injections, elution 6 min 5% B, 18 min 5-35% B, 27 min 35-45% B). The material in the product peak was desalted to give 27.6  $A_{260}$  units (0.406  $\mu$ mole, overall yield 7.8%,  $\lambda$  max 268 nm, 260/280 1.265 calc. 1.298).  $\mu$ -Bondapak C18 hplc assay showed 95% purity. The sequences of both octanucleotides were confirmed by standard analysis.

The Dodecanucleotides d(T-T-C-C-C-A-C-C-A-T-T-T) and d(C-T-C-C-C-A-C-C-A-T-T-T). Resin (0.354 g, 0.109 mmole, Glycine) was derivatised as described above to give the 5'-O-dimethoxytritylthymidine-3'-O-succinamido resin (trityl 0.250 mmole g<sup>-1</sup>, trityl/gly 0.97). Using the manual solvent delivery system two cycles of assembly were carried out with (MeO)<sub>2</sub>TrdT-(ClPh) in the coupling steps. Trityl/gly values at each stage were 0.82 and 0.81 respectively. Using part of the resin (0.237 g, 0.049 mmole) two further cycles of assembly were carried out with the trinucleotide block (MeO)<sub>2</sub>TrdbzC<sup>O</sup>-bzC<sup>O</sup>-bzA-(ClPh) in the coupling steps followed by one cycle with the dinucleotide block (MeO)<sub>2</sub>TrdT<sup>O</sup>-bzC-(ClPh). The resin was divided into two approximately

equal portions. With each portion one cycle of assembly was carried out using  $(\text{MeO})_2\text{TrdT}-(\text{ClPh})$  in the coupling step in one case and  $(\text{MeO})_2\text{TrdbzC}-(\text{ClPh})$  in the other. Isolation of oligonucleotides at various stages of synthesis is summarised in Table 3. Chromatography in all cases was carried out on Partisil 10SAX. Elution conditions for the dodecanucleotides (Figs. 10,11) were 6 min 10% B, 20 min 10-65% B, 25 min 65-90% B. The sequences of both dodecanucleotides were confirmed by standard analysis of  $^{32}\text{P}$ -labelled samples.

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#### Appendix

Oligonucleotide sequencing data and  $\mu$ -Bondapak C18 chromatography profiles were supplied to the referees but are omitted from the paper because of space limitation. The material is available on request.

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**Rapid synthesis of oligodeoxyribonucleotides VI. Efficient, mechanised synthesis of heptadecadeoxyribonucleotides by an improved solid phase phosphotriester route**

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**ABSTRACT**

Efficient mechanised synthesis of heptadecadeoxyribonucleotides has been achieved on an economically small scale by an improved solid phase phosphotriester method on a polydimethylacrylamide resin. Improvements were made in the preparation of dinucleotide building blocks, reaction conditions for oligonucleotide assembly and in purification of deprotected oligonucleotides by h.p.l.c. Several milligrams of pure heptadecamers were obtained. Two of the heptadecamers were designed for sequencing in opposite directions of DNA cloned in phage M13mp2.

**INTRODUCTION**

We have previously demonstrated the practical utility of polydimethylacrylamide resins for solid phase synthesis of oligodeoxyribonucleotides<sup>1-3</sup>. Our recent phosphotriester assembly strategy involved the coupling of appropriate monomer, dimer or trimer blocks to the support in pyridine in the presence of a coupling agent<sup>2,3</sup>. By this route we were able to rapidly prepare oligomers up to 12 units long containing all four bases.

A number of other polymers have also been recommended for use with a phosphotriester strategy, viz. polyacryloylmorpholide<sup>4,5</sup>, cellulose<sup>6</sup> and polystyrene copolymers<sup>7</sup>. It was shown that the use of polyamide resins is preferable to polystyrene in that the yield in the first coupling step to the support is much better<sup>7</sup>. None of these other polymers has yet been used in mechanised oligonucleotide assembly systems. Alternative synthetic routes have been prepared that do not involve use of coupling agents. Good coupling yields were obtained by the addition of deoxynucleoside-3'-O-chlorophenylphosphotriazolidines to a polystyrene support suspended in tetrahydrofuran in the presence of N,N-dimethylaminopyridine<sup>8</sup>. Similarly good results were obtained using a 'phosphite-triester' method with silica gel as support<sup>9</sup>. As yet only monomer units have been coupled to supports by these last two methods and the longest oligonucleotide containing all four bases so far reported made is 13

long.

Recently Markham *et al.* have described the synthesis of oligodeoxyribonucleotides up to 21 long on a polydimethylacrylamide resin using our original phosphotriester route<sup>10</sup>. We now describe a number of significant improvements to our strategy and the mechanised assembly and rapid isolation of heptadecadeoxyribonucleotides on an economically small scale and in substantially better yields than hitherto. The oligonucleotides d(G-T-A-A-A-C-G-A-C-G-G-C-C-A-G-T) and d(C-A-G-G-A-A-C-A-G-C-T-A-T-G-A-C) were designed as primers for the sequencing of DNA cloned in bacteriophage M13mp2<sup>11,12</sup>; the heptadecamer d(G-T-A-T-T-T-T-A-C-A-A-C-A-A-T-T) corresponds to part of the 5' end of tobacco mosaic virus RNA and the heptadecamer d(G-C-A-G-C-C-T-G-A-G-A-G-T-A-G-C-T) was designed as a probe for the cDNA corresponding to the mRNA of the histocompatibility antigen HLA.B8.

#### DISCUSSION

The basic phosphotriester assembly strategy using a beaded polydimethylacrylamide resin<sup>13</sup> has been outlined previously<sup>2,3</sup>. Synthesis is carried out in a 3'-5' direction and the 3'-terminal residue is attached to the support via an alkali-labile 3'-O-succinate linkage. We have found that resin functionalised with each of the four 5'-O-dimethoxytrityl-2'-deoxynucleoside derivatives may be conveniently prepared on a 1 g scale as previously described<sup>2</sup> and stored as dry beads at -20° for at least nine months without noticeable decomposition. Each resin contains 0.22-0.28 mmole g<sup>-1</sup> of deoxynucleoside derivative and a trityl/glycine ratio of 0.90-0.99 (the resin contains an internal glycine marker as previously described<sup>2,3</sup>).

The resin is swollen in pyridine and treated with 10% phenylisocyanate in pyridine as a precaution to ensure the absence of water. Oligonucleotide assembly is carried out by successive cycles of synthesis that involve (1) acidic removal of terminal 5'-O-dimethoxytrityl groups and (2) reaction of the liberated hydroxyl groups with 5'-O-dimethoxytrityl-2'-deoxynucleoside- or oligonucleotide-3'-O-p-chlorophenylphosphates in the presence of a coupling agent (Fig. 1). These synthetic cycles are carried out in a totally enclosed 'oscillating' glass reaction vessel fitted with a sintered glass disc<sup>1,2</sup>, using either a bench-top valve/solvent bottle system<sup>1</sup> or a modified Beckman 990.8 Solid Phase Peptide Synthesiser<sup>1,2</sup>. Although equally good oligonucleotide assemblies may be obtained using either system, we have concentrated particularly on the use of the mechanised Synthesiser, since its use reduces both the time and labour of assembly. Also other 'oscillating vessel' Synthesisers are

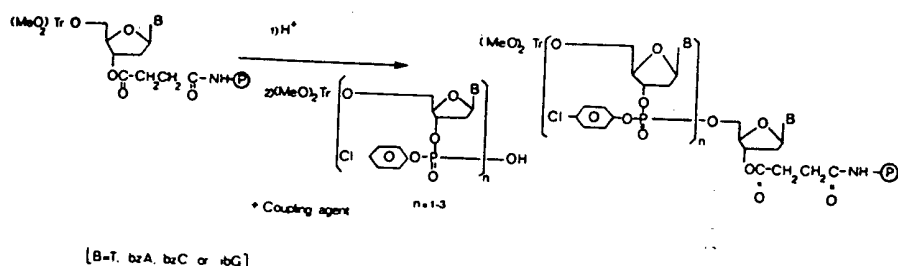


Figure 1

now commercially available<sup>14</sup>.

Since relatively small quantities of oligonucleotides are sufficient for most biological applications we decided for reasons of economy to reduce the scale of synthesis to a minimum. The smallest quantity of resin that can be conveniently handled in practice is 50-60 mg (14  $\mu$ mole of functional group) and we have, therefore, adapted our methods to suit this scale.

#### Preparation of protected mono- and dinucleotides

For the synthesis of oligonucleotides up to about 20 long the maximum use of dimers (rather than trimers) represents to us the best strategy. This keeps the number of reaction cycles to 10 or less and only 20 units (four monomers, 16 dimers) are necessary in order to be able to prepare any desired sequence. One advantage of small scale oligonucleotide assembly is that 1 g of a fully protected dimer is sufficient for at least 10 reaction cycles. We have therefore prepared on a 1-2 g scale all 16 dinucleotides of the form,  $(\text{MeO})_2\text{TrdX}^{\text{O}}\text{X}'^{\text{O}}(\text{CE})$  where X and X' represent 2'-deoxyribonucleosides, *viz.* 2'-deoxythymidine, 6-N-benzoyl-2'-deoxyadenosine, 4-N-benzoyl-2'-deoxycytidine or 2-N-isobutyryl-2'-deoxyguanosine, and  $\text{O}$  represents a p-chlorophenyl protected 3'-5' phosphodiester. To this end we have utilised the 'barium salt' route of Cough *et al.*<sup>15</sup>, but with a number of important improvements (Fig. 2).

(1) The nucleotide barium salts (I) may be conveniently prepared on a 10 mmole scale by phosphorylation of the 5'-O-dimethoxytrityl-2'-deoxynucleoside derivatives in pyridine with a prereacted solution of 1.85 equivalents of p-chlorophenylphosphodichloridate and eight equivalents of triazole in pyridine. No triethylamine is used in the prereaction and hence no filtration step is required for removal of triethylammonium chloride. The reaction is complete within 10 min and in contrast to the observations of Broka *et al.*<sup>16</sup>

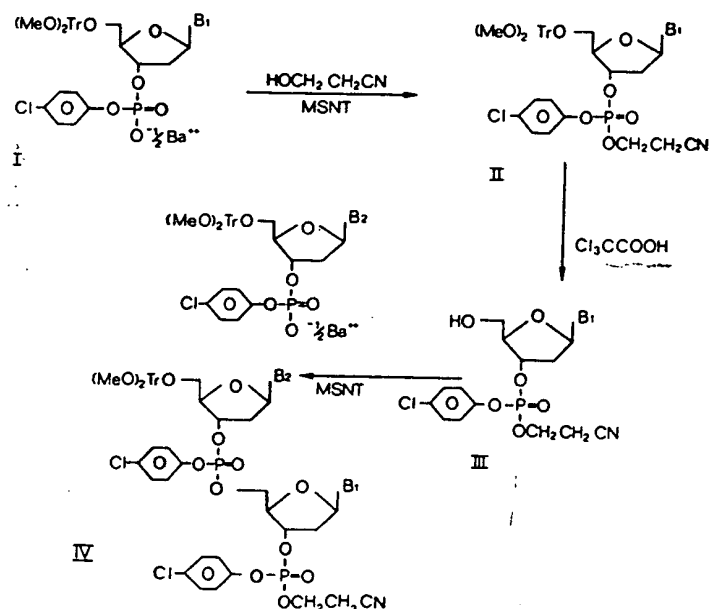


Figure 2

no side reactions involving the guanine moiety or other bases are seen under these conditions<sup>17</sup>. Subsequent hydrolysis and precipitation of the barium salt is carried out essentially as described by Gough *et al.*<sup>15</sup>.

(2) The coupling agent 1-(mesitylene-2-sulphonyl)-3-nitro-1,2,4-triazole (MSNT)<sup>18</sup> is used for both the preparation of the fully protected phosphotriester II and for coupling to form the dimer IV. In general these reactions are complete within 1 h and a minimum of side products is observed<sup>19</sup>.

(3) Removal of 5'-O-dimethoxytrityl groups is accomplished by reaction with three equivalents of trichloroacetic acid in chloroform at 0°. There is less danger of loss of N-benzoyladenine from 6-N-benzoyl-2'-deoxyadenosine derivatives using this reagent rather than benzenesulphonic acid<sup>3</sup>.

(4) After neutralisation of trichloroacetic acid with pyridine all four monomer units of type III may be efficiently extracted into chloroform from 0.1 M sodium bicarbonate solution (*cf.* Gough *et al.* found it impossible to completely extract the deoxyguanosine derivative into ethyl acetate and hence a more complicated protocol was needed for these derivatives<sup>15</sup>).

(5) The use of short column chromatography<sup>20</sup> using silica gel H we find

gives much better separations than our previous system. 1-2 g of dimer may be separated on a 35 g silica column in 1-2 h using 800-1000 ml of solvent (ethanol/chloroform) under slight nitrogen pressure.

Yields of dimers were 54-84% based on the hydroxy component III and products were homogeneous on silica gel tlc. Prior to oligonucleotide assembly 75-80  $\mu$ mole quantities of the appropriate dimers are treated with anhydrous triethylamine/acetonitrile (1:1) for 1 h to remove terminal cyanoethyl groups<sup>3</sup> and the dimers isolated by precipitation into diethyl ether.

For the preparation of oligonucleotides containing an even number of bases a single monomer unit is required in the first assembly cycle. We have confirmed the findings of Norris *et al.*<sup>5</sup> that efficient couplings to the resin may be obtained using the barium salt of the appropriate nucleotide derivative I.

#### Improvements to the assembly strategy

(1) Removal of terminal dimethoxytrityl groups. We have already shown that the use of trichloroacetic acid is preferable to benzene sulphonic acid for removal of dimethoxytrityl groups, especially when 6-N-benzoyl-2'-deoxyadenosine derivatives are present<sup>3</sup>. When our previously described conditions, 10% trichloroacetic acid in chloroform/methanol (98:2) were used on small scale, intermittent failures in coupling reactions were observed. It was found that small droplets of water were slowly formed on storage of the solution of acid, presumably due to esterification of trichloroacetic acid by methanol. This water was retained by the resin during deprotection and then inhibited subsequent coupling reactions. This problem was eliminated by use of 10% trichloroacetic acid in chloroform alone. 2 x 2 treatments at room temperature are sufficient for complete reaction in all cases which is then quenched by addition of dimethylformamide.

(2) Internucleotide coupling reactions. In a model reaction in solution it was shown that the use of MSNT led to faster coupling rates than TPS tetrazole, which we had previously used, and that unwanted 5'-O-sulphonation was only ca. 1% when the concentration of coupling agent was 0.1 M<sup>21</sup>. We have now found that MSNT is a convenient coupling agent for use in solid phase synthesis. Time for coupling is reduced to 90 min and the overall cycle time is reduced to 3-3½ h. (N.B. The cycle time has not been totally optimised and a substantial reduction may be possible in times for intermediate washings.)

Many other solid phase routes described recently have incorporated a co-evaporation step prior to coupling to remove traces of water<sup>4-7</sup>. In

## Nucleic Acids Research

mechanised assembly this technique is cumbersome and slow. However, relative small amounts of water (2-3  $\mu$ l) could totally inhibit coupling reactions on small scale. We have found that good coupling reactions are consistently achieved by addition of an extra quantity of coupling agent in the coupling reaction (five equivalents of dimer and 15 equivalents of MSNT per equivalent of resin functionality). The use of excess coupling agent is not detrimental provided that oximate deprotection is used at the end of assembly to reverse any modifications on guanine residues<sup>19</sup>.

The synthetic cycle (Experimental section) is fully programmed on the Synthesiser and manual intervention is required only when activated monomer or dimer is added (step 8) or to obtain resin samples after each cycle for trityl analysis<sup>2,3</sup>.

(3) Deprotection and isolation of oligonucleotides. The resin is reacted with 1,1,4,4-tetramethylguanidinium syn-p-nitrobenzaldoxime in dioxan/water (1:1)<sup>22</sup> for 60 h. We have found that for long oligonucleotides a shorter time of reaction can give rise to incomplete removal of p-chlorophenyl groups and or incomplete reversal of base-modifications. This leads to internucleotide cleavage and irreversible base-modification respectively during subsequent steps. The oligonucleotide is further deprotected using concentrated ammonia at 50° for 5 h followed by 80% aqueous acetic acid for 30 min at room temperature<sup>2,3</sup>.

Initial purification is achieved by ion exchange h.p.l.c. on Partisil 10 SAX. Markham *et al.* have reported that resolution of long oligonucleotides was not obtained using 5% ethanol in aqueous buffer systems<sup>10</sup>. We have now found that excellent resolution is obtained at least up to the 17-mer level by use of gradients of potassium phosphate (pH 6.2) in the presence of 30% ethanol and at 50-60°. After desalting on Biogel P2 the oligonucleotide is further chromatographed on  $\mu$ -Bondapak C18. We confirm the observations of Markham *et al.* that recovery yields are sometimes worse than expected<sup>10</sup>, but in our experience there is no direct correlation with oligonucleotide length.

The significant improvements to the methodology of solid phase synthesis are exemplified below.

### Synthesis of heptadecanucleotides

Primers for sequencing of cloned DNA in phage M13mp2; d(G-T-A-A-A-C-G-A-C-G-G-C-C-A-G-T) and d(C-A-G-G-A-A-C-A-G-C-T-A-T-G-A-C). Anderson *et al.* have shown the utility of the cloned primer psp14 for sequencing cloned DNA in phage M13mp2<sup>23</sup>. One strand of this primer (primer 1) had 24 bases complementary to the region just prior to the *Eco*RI site (Fig. 3). However, a

## M13 SEQUENCING PRIMERS

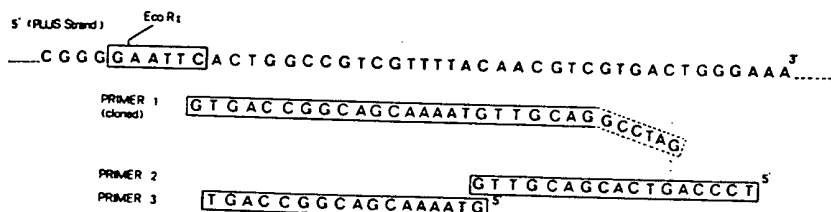


Figure 3

synthetic primer has the advantage that it may be prepared on considerably larger scale. Hence, the oligonucleotide d(T-C-C-A-G-T-C-A-C-G-A-C-G-T-T-G), primer 2, was synthesised by the methods described in our previous paper<sup>3,24</sup>. Unfortunately this primer had a secondary binding site on M13 that resulted in the presence of artifact bands on autoradiographs of sequencing gels. The artifacts could not be completely eliminated in all cases even under conditions of template excess<sup>25</sup>. Analysis of the M13mp2 (+) strand sequence<sup>26</sup> using the computer program SEQFIT<sup>27</sup> showed 13 out of 17 bases exactly complementary at the secondary site. A new primer was chosen by comparing all possible 17-mers with the M13mp2 sequence and eliminating all those in which more than 11 nucleotides were complementary at a second site. Of the remaining sequences, all of which scored a match of 11, the 17-mer, d(G-T-A-A-A-C-G-A-C-G-C-C-A-G-T), primer 3, was chosen as this minimised any complementarity at the 3' end of the primer<sup>28</sup>. The 17-mer has now been shown to be excellent for sequencing DNA in phage M13mp2<sup>11,12,25</sup>.

The oligonucleotide d(C-A-G-G-A-A-C-A-G-C-T-A-T-G-A-C) was designed as a primer for sequencing in the reverse direction DNA cloned in phage M13mp2. The primer has been successful both to confirm sequences derived from sequencing in the normal direction and to obtain the sequences of much longer pieces of DNA. Full details of the use of this primer are published separately<sup>11</sup>.

The syntheses were carried out as described above using solely dinucleotides as basic units, except that in the case of the first oligonucleotide only 10 equivalents of MSNT were used in the coupling step. Thus a larger number of shorter oligonucleotides were observed in the h.p.l.c. elution patterns (Fig. 4) due to incomplete couplings and the overall yield was only 1.3% (Table 1). The second 17-mer was isolated in 5.0% yield and the h.p.l.c.

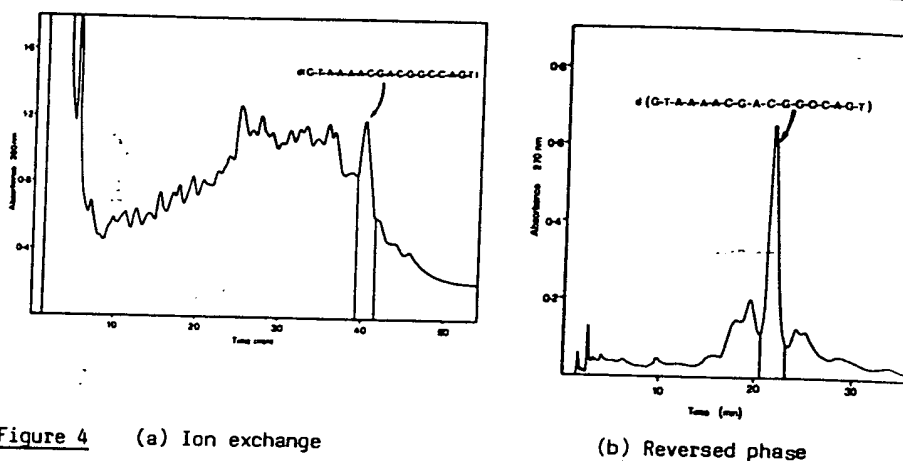


Figure 4 (a) Ion exchange

(b) Reversed phase

elution patterns were much better (Fig. 5).

The heptadecanucleotides, d(G-T-A-T-T-T-T-A-C-A-A-C-A-A-T-T) and d(G-C-A-G-C-C-T-G-A-G-A-G-T-A-G-C-T). The 17-mer, d(G-T-A-T-T-T-T-T-A-C-A-A-C-A-A-T-T), corresponds to part of the 5' end of tobacco mosaic virus RNA and has been used as an aid in cloning of cDNA<sup>29</sup>. The 17-mer, d(G-C-A-G-C-C-T-G-A-G-A-G-T-A-G-C-T), was designed as a probe for the cDNA corresponding to the mRNA of the histocompatibility antigen HLA.B8<sup>30</sup>. The syntheses were carried out as described above, once again using dimers as basic units, and the 17-mer isolated in 7.5% and 9.2% yields respectively (Table 1). H.p.l.c. elution patterns are shown in Figures 6 and 7.

The sequences of all oligonucleotides have been confirmed by standard analysis of <sup>32</sup>P-labelled samples.

TABLE 1

Sequence	Product isolated		Overall yield %	Yield after $\mu$ -Bondapak	
	A <sub>260</sub>	$\mu$ mole		A <sub>260</sub>	% recovery
d(GTAAAACGACGGCCAGT)	35.0	0.180	1.3	8.6	25
d(CAGGAAACAGCTATGAC)	142.0	0.705	5.0	53.0	37
d(GTATTTTACAACAATT)	198.0	1.047	7.5	88.0	44
d(GCAGCCTGAGAGTAGCT)	240.0	1.280	9.2	137.0	57



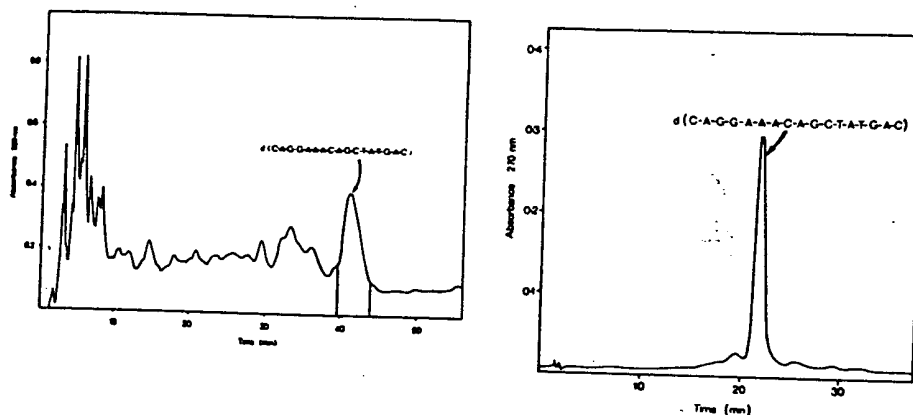


Figure 5 (a) Ion exchange

(b) Reversed phase

## CONCLUSIONS

We have shown that oligodeoxyribonucleotides of 17 residues may be prepared on an economically small scale, in good overall yields (5-9%) and in sufficient quantities for most biological purposes (1-10 mg). Average overall yields per step are 70-75%, but it is clear from h.p.l.c. patterns that coupling yields are substantially higher than this. Use of a mechanised synthesiser allows 2-3 reaction cycles to be readily carried out per day and oligonucleotides may be routinely prepared and isolated in pure form within two weeks. No direct comparison is possible with results obtained with phosphotriester syntheses on other supports, since in no cases have overall

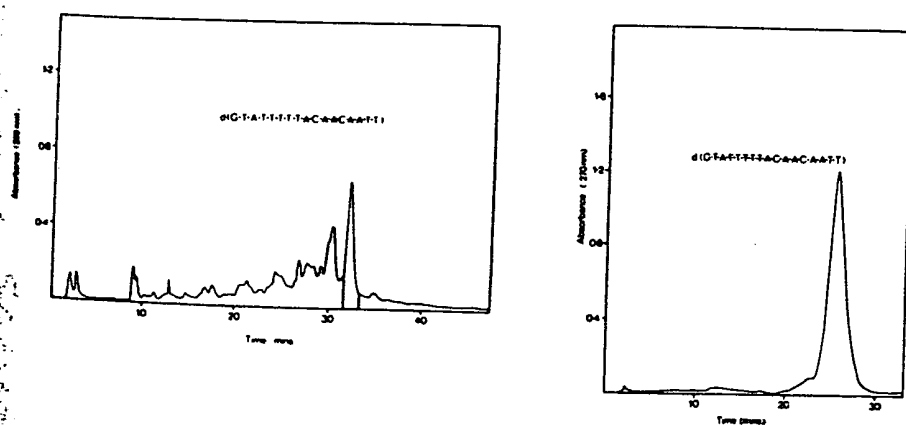


Figure 6 (a) Ion exchange

(b) Reversed phase

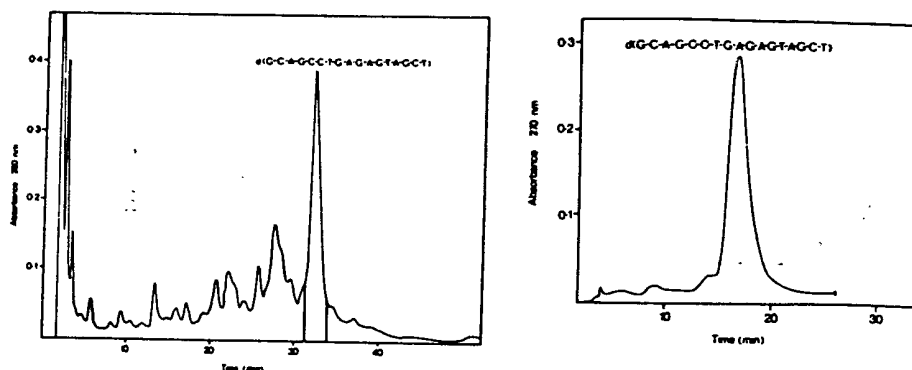


Figure 7 (a) Ion exchange

(b) Reversed phase

isolated yields of final oligonucleotide been quoted<sup>2-9</sup>. It is noteworthy, however, that our overall yields compare favourably with available solution methodologies<sup>31</sup>.

Future work is aimed at further increasing yields and extending the range of oligonucleotides attainable. Analysis of resin eluates after acidic deprotection steps in synthesis of the 17-mer, d(G-I-A-I-I-I-I-T-A-C-A-A-C-A-A-I-I), still showed a total of 27% loss of N-benzoyladenine during the complete synthesis (i.e. ~1% depurination per adenine per deprotection step). Very recently we have found that depurination can be reduced still further if trichloroacetic acid treatment is carried out at 0°C<sup>32</sup>. The use of O-chlorophenyl as protecting group for phosphodiester<sup>4,8,18</sup> is also under investigation. We have already confirmed that dinucleotide blocks incorporating this protecting group may be readily prepared by the route described herein<sup>33</sup>.

#### EXPERIMENTAL SECTION

Unless otherwise mentioned, materials and methods are as previously described<sup>1-3</sup>. Silica gel chromatography was carried out on Kieselgel 60H (Merck 7736) by the short column method<sup>20</sup>. Typically 1-2 g of product dissolved in 10-20 ml of chloroform/0.1% pyridine was applied to 35 g silica in a column fitted with a flat sintered glass disc (diameter 60 %) and eluted under slight nitrogen pressure with ethanol/chloroform containing 0.1% pyridine. The silica was used for one separation only. Ion exchange h.p.l.c. was carried out as previously described<sup>2</sup> except that buffer A was 1 mM potassium phosphate (pH 6.2)/30% ethanol and buffer B was 0.3 M potassium

phosphate (pH 6.2)/30% ethanol. Columns of Partisil 10 SAX were operated at 50-60° at flow rates of 2 ml min<sup>-1</sup> (analytical) or 7 ml min<sup>-1</sup> (preparative). Reverse phase chromatography was carried out on  $\mu$ -Bondapak C18 (Waters) at ambient temperature as previously described<sup>2,3</sup>. Samples were warmed to 50° before injection. Buffer A was 0.1 M NH<sub>4</sub>OAc, buffer B was 0.1 M NH<sub>4</sub>OAc/CH<sub>3</sub>CN (2:8).

Preparation of barium 5'-O-dimethoxytrityl-2'-deoxynucleoside-3'-p-chloro-phosphates

Triazole (5.5 g, 80 mmole), recrystallised from anhydrous dioxan, was coevaporated twice with anhydrous pyridine and finally dissolved in pyridine (50 ml). p-Chlorophenylphosphodichloridate (3.7 ml, 18.5 mmole) was added and the mixture left at room temperature for 15 min. Meanwhile in a separate flask the 5'-O-dimethoxytrityldeoxynucleoside derivative of dI, dbzA, dibG or dbzC (10 mmole) was coevaporated three times with anhydrous pyridine leaving a final volume of 100 ml. To this was added the phosphorylating mixture and after 10 min the solution was added slowly to vigorously stirred water (500 ml). A clear solution soon resulted and after 30 min this was poured slowly into a vigorously stirred, ice-cold solution of barium chloride (20 g of the dihydrate in 2 l of water). The mixture was stirred for 30 min. If a filterable precipitate had not been achieved the mixture was warmed to 30° with continual stirring and once more cooled to 0°. The precipitate was collected in a coarsely sintered glass funnel using gravity or very slight water pump pressure. After washing with a little iced water the precipitate was dried to constant weight over P<sub>2</sub>O<sub>5</sub>. Yields 80-90%. The product may still contain several moles of water.

General procedure for preparation of fully protected dimers (1.5 mmole scale)

(a) Preparation of the hydroxyl component. The barium 5'-O-(MeO)<sub>2</sub>Tr-deoxynucleoside-3'-p-chlorophenylphosphate (6.6 mmole) was coevaporated three times with pyridine to give a final volume of 65 ml. 3-hydroxypropionitrile (Aldrich, stored over mol. sieve 4A) (2.2 ml, 32.2 mmole) was added followed by mesitylenesulphonyl-3-nitro-1,2,4-triazole (MSNT) (3.46 g, 13 mmole). After 1 h at room temperature silica gel tlc in 10% ethanol/chloroform showed almost complete conversion to a spot Rf 0.5-0.7. Ethyl acetate (450 ml) was added and the mixture washed with 1 M NaHCO<sub>3</sub> solution (3 x 150 ml) and then saturated sodium chloride solution (150 ml). The organic phase (and any necessary backwashings) was evaporated to an oil with pyridine and then coevaporated twice with toluene. The product was dissolved in chloroform (50 ml) and cooled in ice. A precooled solution of trichloroacetic acid (3.19

## Nucleic Acids Research

g, 19.5 mmole) in chloroform (50 ml) was added and the dark orange solution left at 0° (the reaction is followed by tlc in 10% ethanol/chloroform and is usually complete within 20 min). Pyridine (10 ml) was added and the solution diluted with chloroform (400 ml) and washed with 0.1 M NaHCO<sub>3</sub> solution (3 x 150 ml) followed by saturated sodium chloride solution (150 ml). The organic phase (and any necessary backwashings) was evaporated to an oil in the presence of pyridine and divided into four portions as pyridine solutions.

(b) Preparation of the dimer. To one of the four portions of 'hydroxyl component' (ca. 1.5 mmole) was added the appropriate barium 5'-O-(MeO)<sub>2</sub>Ir-deoxynucleoside-3'-O-p-chlorophenylphosphate (2 mmole) and the mixture coevaporated three times with pyridine to give a final volume of 20 ml. MS<sup>1</sup> (1.06 g, 4 mmole) was added and the solution left at room temperature for 1 h. Silica gel tlc in 10% ethanol/chloroform showed complete conversion of the hydroxyl component to higher R<sub>f</sub> spot (or spots). Water (10 ml) was added and after 20-30 min chloroform (200 ml) was added and the mixture washed with 0.1 M NaHCO<sub>3</sub> solution (3 x 150 ml) followed by saturated sodium chloride solution (150 ml). On occasion emulsions were formed which were separated by centrifugation. The organic phase (and any necessary backwashings) was evaporated to an oil with pyridine, diluted with chloroform to ca. 15 ml and chromatographed on silica gel H (35 g) by the short column method. The column was eluted with 300 ml of chloroform/0.1% pyridine followed by a % of ethanol in chloroform/0.1% pyridine (see below) under ca. 5 psi pressure of nitrogen. The eluate was monitored by tlc and pure fractions pooled, evaporated to an oil with pyridine, dissolved in chloroform and product precipitated with ether/pentane (1:2). Yields 54-84% (based on hydroxyl component). Percentages of ethanol required in column elution: TT,TA-2.5%; TC,CC,CA,CT,AC,AA,AT-3%; TG,CG,GT,GA,GC-4%; AG,GG-5%.

### Removal of cyanoethanol groups from dimers

The dimer (75-80 μmole) was dissolved in triethylamine/acetonitrile (1:2 ml) in a 10 ml flask. The reaction was followed by tlc in 10% ethanol/chloroform and was usually complete within 1 h to give one spot on the baseline. The solution was evaporated to an oil under high vacuum, dissolved in 2-3 ml chloroform/0.1% pyridine and product precipitated by dropwise addition to 200 ml anhydrous diethyl ether vigorously stirred in a centrifuge tube. The precipitate was separated by centrifugation and decantation, washed with 2 x 100 ml ether and dried in vacuo. Yields 90-95%.

### Oligonucleotide assembly

The resin (14 μmole functionality) contained in a glass reaction

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vessel<sup>1,2</sup>, is swollen in pyridine overnight and washed with (1) 5 x pyridine, 2 min, (2) 1 x 10% phenylisocyanate/pyridine, 30 min, (3) 5 x pyridine, 2 min, followed by the appropriate number of synthetic coupling cycles (Table 2) using a modified Beckman 990B solid phase peptide synthesiser<sup>1-3</sup>. Delivery volume per wash is 5-6 ml.

### Deprotection and cleavage from the resin

The resin is shaken with 0.3 M tetramethylguanidinium p-nitrobenzaldehyde in dioxan/water (1:1) (5 ml) for 60 h, the liquid decanted and resin washed with dioxan/water (3 x 5 ml). The decantate and washings are carefully neutralised with acetic acid, washed once with chloroform and the aqueous phase evaporated to dryness. The residue is treated with concentrated ammonia (10 ml) at 50° for 5 h in a sealed flask, evaporated to dryness and treated with acetic acid/water (4:1, 10 ml) for 30 min. The solution was washed three times with diethyl ether and evaporated to dryness. Product was dissolved in water ready for h.p.l.c.

### Purification of oligonucleotides

1. d(G-T-A-A-A-C-G-A-C-G-G-C-C-A-G-T). Ion exchange: 89% injected in eight portions, 60°, 4' 30% B, 50' 30-90% B; desalted on Biogel P2; Reverse phase in

TABLE 2

Step	Reagent or Solvent	Time of Shaking (min)	No. of Operations
1	CHCl <sub>3</sub>	2	10
2	CHCl <sub>3</sub>	5	3
3	10% TCA/CHCl <sub>3</sub>	2	2
4	DMF	2	1
5	CHCl <sub>3</sub>	2	5
6	DMF	2	5
7	Pyridine	2	10
8	Coupling Mixture <sup>†</sup>	90	1
9	Pyridine	2	5

The deoxynucleoside- or oligonucleotide-3'-p-chlorophenylphosphate (70 μmole) is dried by coevaporation three times with anhydrous pyridine to a final volume of 1.5 ml. MSNT (210 μmole) is added and the resultant solution quickly added to the resin by means of a Pasteur pipette.

## Nucleic Acids Research

seven portions, 4' 8% B, 45' 8-15% B. Yield: 8.6  $A_{260}$  units, 0.045  $\mu$ mole - see Table 1.

2. d(C-A-G-G-A-A-A-C-A-G-C-T-A-T-G-A-C). Ion exchange: eight portions, 65°, 4' 40% B, 45' 40-80% B; desalted on Biogel P2; Reverse phase in seven portions 4' 8% B, 45' 8-15% B. Yield: 53  $A_{260}$  units, 0.263  $\mu$ mole - see Table 1.

3. d(G-T-A-T-T-T-T-T-A-C-A-A-C-A-A-T-T). Ion exchange: eight portions, 54°, 4' 30% B, 30' 30-65% B (example shown: 4' 0% B, 45' 0-90% B); desalted on Biogel P2; Reverse phase: nine portions, 35°, 4' 10% B, 40' 10-13% B. Yield: 88  $A_{260}$  units, 0.465  $\mu$ mole - see Table 1.

4. d(G-C-A-G-C-C-T-G-A-G-A-G-T-A-G-C-T). Ion exchange: ten portions, 50°, 4' 30% B, 50' 30-90% B; desalted on Biogel P2; Reverse phase, ten portions, 4' 9% B, 45' 9-13% B. Yield: 137  $A_{260}$  units, 1.280  $\mu$ mole - see Table 1.

### $^{32}$ P-Labeling and sequencing

The four heptadecamers were  $^{32}$ P-labelled using  $\gamma$ - $^{32}$ P-ATP and T4 polynucleotide kinase under standard conditions and run on a 20% polyacrylamide gel (0.3%) in the presence of 7 M urea and 0.1 M Tris-Borate (pH 8.3), 2 mM EDTA (Fig. 8). All showed single bands which were eluted in water and sequenced by wandering spot analysis. All showed the expected pattern of spots<sup>34</sup>.

Marker 1 2 3 4 ← Origin

← Slow Blue

← Fast Blue

← ATP

**Figure 8**

20% polyacrylamide gel of reaction mixture from  $^{32}$ P-labelling of:

- 1) d(G-T-A-A-A-A-C-G-A-C-G-G-C-C-A-G-T),
  - 2) d(C-A-G-G-A-A-A-C-A-G-C-T-A-T-G-A-C),
  - 3) d(G-T-A-T-T-T-T-T-A-C-A-A-C-A-A-T-T),
  - 4) d(G-C-A-G-C-C-T-G-A-G-A-G-T-A-G-C-T),
- and MARKER: d(T-C-C-C-A-G-T-C-A-C-G-A-C-G-T-T-G)

ACKNOWLEDGEMENTS

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\* to whom reprint requests should be sent

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SEMIAUTOMATED SYNTHESIS OF OLIGONUCLEOTIDES  
ON A SILICA GEL SUPPORT

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Dodecadeoxynucleotides have been synthesized by the phosphotriester method on a column of  $\gamma$ -aminopropyl silica gel or benzylaminopolystyrene by using a programmed synthesizer.

Polymer support syntheses of oligodeoxyribonucleotides have been improved by several groups using the phosphotriester<sup>1-3)</sup> and phosphite<sup>4-6)</sup> methods. Polyacrylamide, polystyrene and silica gel are the most commonly used in the phosphotriester or phosphite approach. Silica gel supports are thought to be suitable to place in a column and have proved to be successful in the phosphite method. In this communication we wish to report the phosphotriester synthesis of a dodecadeoxynucleotide by condensation of mononucleotides on a column of silica gel using a programmed machine. A similar reaction was performed on a polystyrene support with a less successful result.

dCATATTCATCGC was synthesized on Porasil C containing  $\gamma$ -aminopropyl group<sup>6)</sup> (0.183 mmol/g) first by condensing the 3'-terminal 5'-dimethoxytrityl-N-benzoyldeoxycytidine 3'-succinyl-pentachlorophenyl ester<sup>2)</sup> (5 fold excess) in DMF in the presence of triethylamine for 24 hr. The scheme is shown in Chart 1. Unchanged amino groups were blocked by treatment with acetic anhydride, pyridine and dimethylaminopyridine (1:9:0.05). The silica gel (106 mg, 10  $\mu$ mol dC content, estimated using  $\epsilon_{499} = 7.17 \times 10^4$  in 3:2 HClO<sub>4</sub>-EtOH) was placed in a column and fitted to a synthesizer (Solid Phase Synthesizer Model 25A, Genetic Design Co., Chart 2). Condensation of 5'-dimethoxytrityl-N-protected nucleoside 3'-(o-chlorophenyl) phosphates was performed as summarized in Table I. The condensation cycle started from the acetic anhydride treatment which removed moisture in the system and blocked unreacted functional groups. The dimethoxytrityl group was removed by treatment with benzenesulfonic acid prior to injection of a mixture of mononucleotides and a condensing reagent (mesitylenesulfonyl 3-nitrotriazolide, MSNT). The yield of each step was estimated by measuring dimethoxytrityl alcohol. It was found that excess of benzenesulfonic acid inhibited the coloring of dimethoxytrityl cations, yields could be underestimated except for the last step. The yield of the last step was estimated by treatment of an aliquot of the support and the result is summarized in Table I.

Removal of the support from the product was performed by treatment with tetramethylguanidium 2-pyridine aldoxamate<sup>7)</sup> in dioxan-water (1:1) for

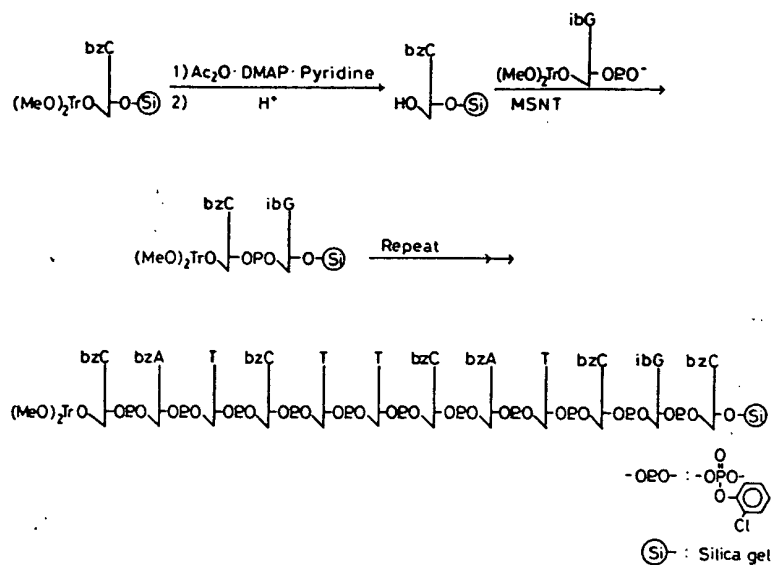


Chart 1

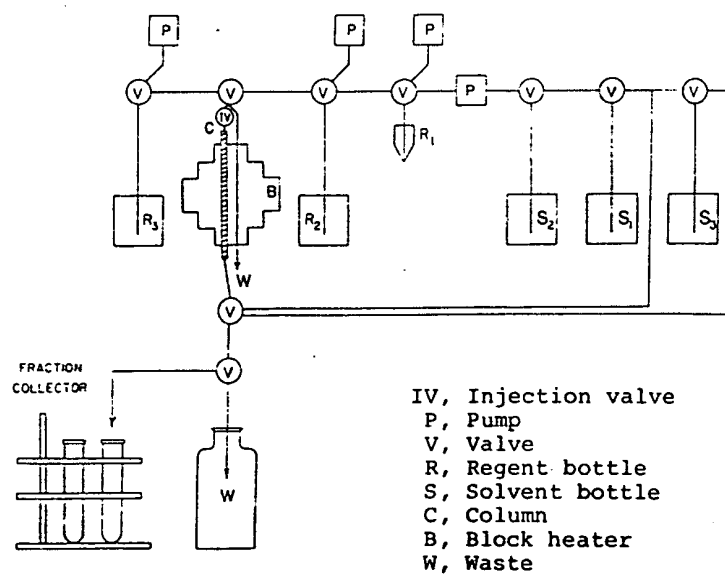


Chart 2

Table I REACTION CYCLE

Step	Solvent or Reagent	Flush, Rest, or Recycle	Collect or Waste	Time (min)
1	Pyridine	flush	waste	5
2	Ac <sub>2</sub> O-DMAP-Pyridine (1:0.05:9)	flush	waste	3
3	—	rest	—	5
4	Ac <sub>2</sub> O-DMAP-Pyridine (1:0.05:9)	flush	waste	3
5	—	rest	—	—
6	Ac <sub>2</sub> O-DMAP-Pyridine (1:0.05:9)	flush	waste	3
7	Pyridine	flush	waste	3
8	CH <sub>2</sub> Cl <sub>2</sub>	flush	waste	10
9	2% BSA in CH <sub>2</sub> Cl <sub>2</sub> -MeOH (7:3)	flush	collect	1
10	CH <sub>2</sub> Cl <sub>2</sub>	flush	collect	1
11	2% BSA in CH <sub>2</sub> Cl <sub>2</sub> -MeOH (7:3)	flush	collect	1
12	CH <sub>2</sub> Cl <sub>2</sub>	flush	collect	5
13	CH <sub>2</sub> Cl <sub>2</sub>	flush	waste	5
14	Pyridine	flush	waste	3
15	N <sub>2</sub>	flush	waste	3
16	Nucleotide & MSNT in Pyridine	rest	—	40
17	—	recycle	—	15
18	Pyridine	flush	waste	5

Table II

Chain length	Sequence of linked oligonucleotide	(MeO) <sub>2</sub> TrOH (A <sub>499</sub> )	Amount (μmol)	Yield (%)	Overall yield (%)
1	HOC-	717	10.0		
2	HOGC-	325	4.53	45.3	45.3
3	HOCGC-	196	2.73	60.3	27.3
4	HOTCGC-	173	2.41	88.3	24.1
5	HOATCGC-	176	2.45	101.7	24.5
6	HOCATCGC-	142	1.97	80.4	19.7
7	HOTCATCGC-	128	1.79	90.9	17.9
8	HOTTCATCGC-	120	1.67	93.8	16.7
9	HOCTTCATCGC-	93.1	1.30	77.8	13.0
10	HOTCTTCATCGC-	86.1	1.20	92.3	12.0
11	HOATCTTCATCGC-	62.1	0.87	72.5	8.7
12	(MeO) <sub>2</sub> TrOCATCTTCATCGC-	68.2	0.95	109.3	9.5

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48 hr. This procedure also removed the o-chlorophenyl groups. The filtered solution was then concentrated and treated with concentrated ammonia to remove N-acyl groups. The 5'-dimethoxytritylated product was separated by reverse phase chromatography (C-18) and deblocked with 80% acetic acid. The deblocked dodecamer dCATCTTCATCGC was further purified by the same chromatography and characterized by mobility shift analysis. Thus the dodecamer was obtained by a simple procedure of mononucleotide condensation in an overall yield of 10% and isolated by a standard procedure of chromatography.

Another dodecamer dCATCTTCATTGC was synthesized by the similar procedure on benzylamino polystyrene <sup>3)</sup> using the same machine. The polystyrene support was mixed with silanized glass beads (20:1) and packed in a column containing the same glass at the bottom. The overall yield was found to be 6 %. Removal of the support from the product was not completed by the procedure described above and the dimethoxytrityl group was detected on the support after oximate treatment. This may mean that more lipophilic condition is necessary for the polystyrene support.

Condensation involving di- and trinucleotides on the silica gel support using the synthesizer were performed and the results will be reported elsewhere.

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RECOVERY AND RECYCLING OF SYNTHETIC UNITS IN THE CONSTRUCTION OF  
OLIGODEOXYRIBONUCLEOTIDES ON SOLID SUPPORTS

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**Summary:** Barium salts of protected mono- and dinucleotides have been employed in a scheme for the construction of oligodeoxyribonucleotides on a glass support. The excesses of these synthetic units were recovered simply by precipitation, and reused in subsequent cycles.

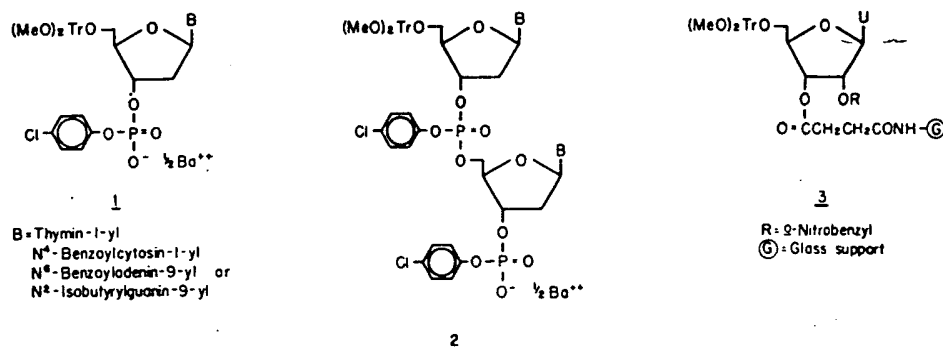
A number of practically useful systems for solid phase synthesis of deoxyribo- and ribo-oligonucleotides have recently been introduced<sup>1-6</sup>. They represent successful adaptations of both phosphotriester<sup>1-4</sup> and phosphite<sup>5,6</sup> methodologies to the construction of oligomers on a variety of supports such as polyacrylamide, polystyrene and silica gel. Whatever the chemistry involved, the basic strategy is the same in all these systems, namely addition of a protected nucleotide monomer or oligonucleotide block to the free 5'-hydroxyl group of a support-linked nucleoside, followed by a deprotection step which, by exposing a new hydroxyl function, allows the cycle to be repeated. The reduction in number of manipulations that results from carrying out these operations on a support rather than in solution is an important advantage, but one significant drawback remains. Since the solid matrix unavoidably "dilutes" the immobilized oligonucleotide chain, larger excesses of the incoming units are required to maintain effective concentrations, and hence reaction rates, relative to the same coupling step in solution. Any method for recovering the unused portions of these units, which are themselves the end-products of multi-step syntheses, would be of considerable value.

We have previously reported<sup>7</sup> the preparation of the barium salts of *N*-protected 5'-O-dimethoxytrityldeoxyribonucleoside-3' *p*-chlorophenyl phosphates [(MeO)<sub>2</sub>Tr]dN'-(ClPh)<sup>8</sup>, (1), which can be isolated in high yield and purity by precipitation from aqueous barium chloride. These monomers, together with the sixteen dinucleotides [(MeO)<sub>2</sub>Tr]dN'<sup>2</sup>dN'<sup>2</sup>(CNEt)<sup>8</sup> are basic building units for our oligonucleotide syntheses carried out in solution. We noted<sup>7</sup> that the fully protected dinucleotides, after decyanoethylation, can also be isolated as the pure barium salts [(MeO)<sub>2</sub>Tr]dN'<sup>2</sup>dN'-(ClPh).4Ba<sup>++</sup> (2).

It occurred to us that the water insolubility of these heavy metal salts might permit their direct recovery from spent reaction mixtures in solid support syntheses simply by pouring them into dilute aqueous barium chloride. However, in the phosphotriester method, such mixtures also contain a condensing agent, and this must be removable in order to isolate the nucleotide salt in pure form. The coupling reagent that we are currently using, 1-(*p*-toluenesulfonyl)-3-nitro-1,2,4-triazole<sup>9</sup>, hydrolyzes rapidly in aqueous pyridine (< 1 hr) to *p*-toluenesulfonate and nitrotriazole, both of which are soluble in dilute barium chloride.

In order to study the feasibility of the recovery procedure we decided to prepare the two ribonucleoside terminated<sup>10</sup> oligomers d(A-T-G-C-A-T)-rU and dC-dT<sub>13</sub>-rU on a glass support. As

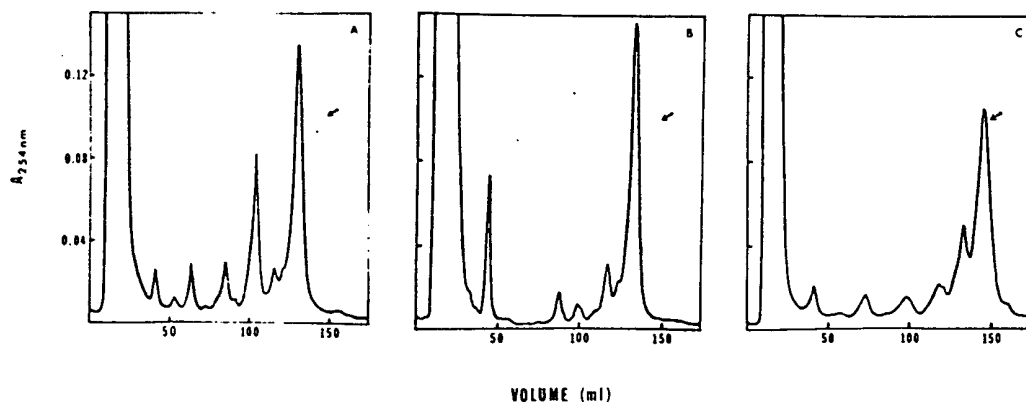
the first step, 5'-O-dimethoxytrityl-2'-O-(o-nitrobenzyl)uridine<sup>11,12</sup> was converted to its 3'-O-(pentachlorophenyl succinate) derivative by the method of Miyoshi *et al.*<sup>13</sup> One gram of long-chain alkylamine controlled pore glass (Pierce Chemical Co., 0.1 mmol amine/g) was then treated with the activated uridine derivative (0.3 mmol) in anhydrous pyridine (3 ml). After degassing, triethylamine (0.43 mmol) was added and the mixture was allowed to stand for 3 days at 25°.



The glass was then washed thoroughly with pyridine and chloroform and dried (1.028 g of 3, R=o-nitrobenzyl). Treatment with isobutyl chloroformate (2 ml added at 0°) in pyridine (30 ml) for 3 days at 25° was carried out to derivatize residual free amino groups. The amount of uridine bound to the glass was determined as 0.041 mmol/g by detritylation and spectrophotometric measurement of the dimethoxytrityl cation<sup>14</sup>. Trityl assay also showed that 80% of the bound nucleoside could be released by 2 days' exposure to tetramethylguanidinium pyridine-2-aldoximate<sup>15</sup> (TMG-PAO, 0.33 M in 50% aqueous dioxane). In each of the syntheses outlined below a sample of the derivatized glass (3, 103 mg, 4.2  $\mu\text{mol}$  of bound uridine) was detritylated, then treated with either 1 or 2 in the presence of *p*-toluenesulfonyl nitrotriazole<sup>16</sup>. When the reaction was complete, the excess reagent was removed, the nucleotide barium salt was recovered for reuse, and the glass was again detritylated ready for the next addition. Details of a complete cycle are given in footnote 17.

The preparation of d(A-T-G-C-A-T)-rU was accomplished in two ways. In the first, monomers (1) were used; the barium salts of  $[(\text{MeO})_2\text{Tr}]dT-(\text{ClPh})$  and  $[(\text{MeO})_2\text{Tr}]dbzA-(\text{ClPh})$ , recovered from the first and second cycles respectively, were used again in the fifth and sixth. The addition yields at each step, estimated from dimethoxytrityl release and expressed as percentages of support-bound uridine, were 91, 89, 86, 64, 91 and 68%, the last being measured from a 5 mg sample of the glass. Barium salts of the monomers were recovered with an average yield of 96%. The oligomer was released from the glass and partially deprotected by 3 days' treatment with 0.33 M TMG-PAO. After a further 3 days in 14 M  $\text{NH}_4\text{OH}$  followed by removal of solvent, the product was treated with 80% AcOH for 25 min, then co-evaporated with water and neutralized. For analysis, 1.5% of the material was chromatographed on Pellionex SAX<sup>18</sup> (0.4x50 cm) using 200 ml of 40% EtOH containing a linear gradient of 0-0.5 M  $\text{NH}_4\text{Cl}$  (pH 8), and the result is shown in

elution pattern A. The overall yield<sup>19</sup> of d(A-T-G-C-A-T)-rU(NBzl), based on the first deoxy-ribonucleotide addition, was 27%. A second synthesis of the same molecule was carried out with dinucleotides 2; the recovered barium salt of [(MeO)<sub>2</sub>Tr]dbzA<sup>2</sup>dT-(ClPh) was reused in the third cycle. Yields in the three cycles were 64, 69, and 76% with respect to U; recovery of the dinucleotides was 99%. The oligomer was released, deprotected, and analyzed as described above (elution pattern B); the overall yield was 39%. The release mixtures from these two syntheses were combined and the d(A-T-G-C-A-T)-rU(NBzl) was isolated by chromatography on Dowex 1-X2 ion-exchange resin<sup>18</sup>. After essentially quantitative removal of the 3' nitrobenzyl group by irradiation with long-wave UV light<sup>12</sup>, the sequence of the heptamer d(A-T-G-C-A-T)-rU was confirmed by mobility shift analysis<sup>20</sup>. Additionally, the terminal uridine residue was removed by treatment with periodate followed by  $\beta$ -elimination<sup>21</sup>. After phosphatase treatment, the resulting hexamer was chromatographically indistinguishable from d(A-T-G-C-A-T) previously synthesized in solution by well-established methods<sup>7, 20</sup>.



The oligomer dC-dT<sub>13</sub>-rU was made by six consecutive additions of [(MeO)<sub>2</sub>Tr]dT<sup>2</sup>dT-(ClPh) to the glass, with recovery and reuse of the dinucleotide barium salt at each step. The addition yields were 64, 71, 57, 57, 57, and 52% and the average recovery of the dinucleotide was 97%. A final reaction with [(MeO)<sub>2</sub>Tr]dbzC<sup>2</sup>dT-(ClPh). $\frac{1}{2}$ Ba<sup>++</sup> went poorly (26% with respect to U), so the glass was given a second 3 hr treatment with this dimer before removal of the dimethoxytrityl group. Following release and deprotection, the pentadecamer dC-dT<sub>13</sub>-rU(NBzl) was obtained in 32% yield (elution pattern C; chromatography was performed as described above except that 1.3% of the total product was used and the solvent was 200 ml of 40% EtOH containing a gradient of 0.1-0.7 M NH<sub>4</sub>Cl). After isolation and removal of the nitrobenzyl group, the material gave the expected pattern on mobility shift analysis.

We conclude by noting that the barium salts of suitably protected mono- and dinucleotides should also be useful for syntheses in the ribo series<sup>11</sup>, on other supports<sup>4</sup>, and in automated systems. In these cases, as in the work described above, recovery and recycling operations can then be used for the conservation of valuable starting materials.

### Acknowledgements

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# Properties of Formaldehyde-Treated Nucleohistone\*

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**ABSTRACT:** When native nucleohistone is treated with formaldehyde, the resulting soluble complex does not dissociate in solutions of high ionic strength and may be banded in a CsCl gradient.

The buoyant density of the complex, 1.411 g/cc, is that calculated from the ratio of deoxyribonucleic acid to histone in nucleohistone. Formaldehyde treatment of nucleohistone cross-links histone molecules to deoxyribonucleic acid.

Thus the histone of the formaldehyde-treated complex is no longer soluble in acid. Treatment with pronase removes 96% of the protein from deoxyribonucleic acid, leaving 4% firmly bound, an amount which is in approximate stoichiometric proportion to the formaldehyde cross-links between deoxyribonucleic acid and protein.

When histones are extracted from nucleohistone with salt and then banded in a CsCl equilibrium density gradient, the width of the resulting band indicates that the banding material consists of aggregates of 10–20 histone molecules rather than of single histone molecules (Huang and Bonner, 1965). In order to determine whether such histone aggregates occur natively in nucleohistone, an attempt was made to attach each histone to its neighbor by treatment of nucleohistone with formaldehyde. We then found that the histones of formaldehyde-treated nucleohistone are not dissociable from DNA by salt. By such treatment histone molecules are firmly bound to DNA. The formaldehyde-stabilized histone-DNA complex is thus similar in its behavior in CsCl to that of formaldehyde-stabilized ribosomes, first studied by Spirin (Spirin *et al.*, 1965). Romakov has studied the reaction of formaldehyde with nucleohistone and has reported on the precipitation properties of the product of the reaction as compared with those of native nucleohistone (Romakov, 1968). In this paper we consider the buoyant density and other properties of formaldehyde-treated nucleohistone.

## Materials and Methods

**Preparation of Nucleohistone.** Chromatin was prepared from pea buds according to the method of Bonner *et al.* (1968) except that the initial grinding media contained 0.25 M sucrose, 0.01 M Tris (pH 8.0), and 0.002 M MgCl<sub>2</sub>. The chromatin pellets from the sucrose purification step were resuspended in 40 ml of 0.01 M triethanolamine (pH 7.8; CalBiochem, heavy metal free) by homogenizing in a Potter-Elvehjem homogenizer. The resulting chromatin solution was dialyzed for 4 hr vs. 0.01 M triethanolamine (pH 7.8). The chromatin was then sheared in the Virtis homogenizer for 90 sec at 40 V, centrifuged at 10,000g for 15 min, and the supernatant was dialyzed overnight vs. 0.01 M triethanolamine (pH 7.8). The resulting nucleohistone usually had a concentration of 20–40 OD<sub>260</sub>, a ratio OD<sub>230</sub>/OD<sub>260</sub> of about 0.7, and showed no tur-

bidity in the 320–360-mμ range. Histone to DNA ratio of the complex was 1.30 as determined by chemical analysis (Lowry *et al.*, 1951).<sup>1</sup> Such nucleohistone constituted our starting material. Preparation of chromatin in the presence of triethanolamine buffer throughout rather than of Tris for the initial steps was unsuccessful in that the chromatin aggregated more readily with contaminating nonchromosomal protein.

**The Formaldehyde Reaction.** The reaction with formaldehyde was performed at various concentrations of formaldehyde, but always at a nucleohistone concentration of 10 OD<sub>260</sub>. The nucleohistone was first diluted to 11.1 OD<sub>260</sub> and then 9 ml of this dilution was added rapidly and with vigorous stirring to 1 ml of a solution of formaldehyde at 10 times the final concentration, buffered pH 7.8 with 0.01 M triethanolamine. The reaction was allowed to proceed for 24 hr at 0°. The solution was then dialyzed vs. 0.01 M triethanolamine (pH 7.8) for 24 hr with at least four changes of buffer.

**Melting Procedure.** The nucleohistone solutions were dialyzed extensively vs. 2.5 × 10<sup>-4</sup> M EDTA (pH 8.0), diluted to 1 OD<sub>260</sub> with dialysate, and then melted in a Gilford Model 2000 multiple-sample absorbance recording apparatus adapted for the recording of melting profiles. The rate of temperature increase was 0.5°/min. All of the melting profiles are normalized to 1.0 OD<sub>260</sub> and are plotted on the same scale for ready comparison. They are not corrected for thermal expansion which is negligible for the present purposes.

**Buoyant Density Determination.** All buoyant density experiments were carried out in the Spinco Model E analytical ultracentrifuge and brought to equilibrium at 44,770 rpm, 25°, for at least 16 hr. The results were recorded using absorption optics employing the Spinco scanner, multiplex, and recorder. The buoyant density was calculated by a computer program, written for the IBM 7094, and based on the method of Ifft *et al.* (1961), which determines the distribution of CsCl throughout the entire cell. This method allows accurate calculation of buoyant density regardless of the isoconcentration density (in the range 1.100–1.800 g/cc) and regardless of the position of the band in the liquid column. The initial concen-

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<sup>1</sup> Care must be taken to eliminate triethanolamine from any sample to be tested with the Lowry reaction as it interferes very strongly even at a concentration of 0.01 M.

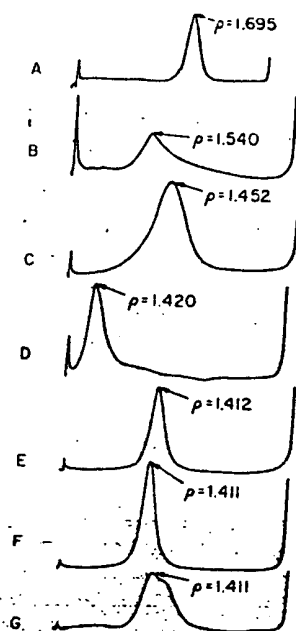


FIGURE 1: Equilibrium buoyant density banding patterns of nucleohistone in CsCl after treatment with various concentrations of formaldehyde. (A) Untreated, buoyant density = 1.695; (B) treated with 0.05% formaldehyde, buoyant density = 1.540; (C) with 0.1% formaldehyde, density 1.452; (D) with 0.2% formaldehyde, density 1.420; (E) with 0.5% formaldehyde, density 1.412; (F) with 1.0% formaldehyde, density 1.411; (G) with 10% formaldehyde, density of main peak is 1.411.

concentration of nucleohistone was 0.1–0.2 OD<sub>260</sub> if the isoconcentration density was 1.400 g/cc or higher, and it was 4–5 OD<sub>260</sub> if the isoconcentration density was in the histone density range.

**Determination of Sedimentation Coefficients.** Sedimentation velocity measurements were made using the band sedimentation method in self-forming density gradients in the analytical centrifuge (Vinograd *et al.*, 1963). D<sub>2</sub>O was used to form the gradients and all sedimentation coefficients were corrected to 5.0 s.w. The partial specific volume of nucleohistone was taken to be 0.650 cc/g as determined by a weight average of the partial specific volumes of DNA (0.555 cc/g) (Brunner and Vinograd, 1965) and of histones (0.745 cc/g).<sup>2</sup>

**Disc Gel Electrophoresis of Histones.** The histone samples were prepared for electrophoresis by adding 0.5 ml of 1 N H<sub>2</sub>SO<sub>4</sub> to 2 ml of nucleohistone at a concentration of 10 OD<sub>260</sub>. The solution was mixed vigorously, allowed to stand at 0° for 15 min, and then centrifuged at 10,000g for 15 min. The supernatant was decanted and mixed with two volumes of cold 100% ethanol and allowed to stand for 30 min. The histones were then precipitated by centrifugation for 15 min at 10,000g. The pellet was washed with 100% ethanol and then air dried. The protein was dissolved in the proper amount of 10 M urea to make the final solution 1 mg/ml. In some cases the amount of acid-extractable protein was determined by the Lowry reaction (Lowry *et al.*, 1951). The standard bovine serum albumin fraction V (Sigma Chemical Co.) was also dis-

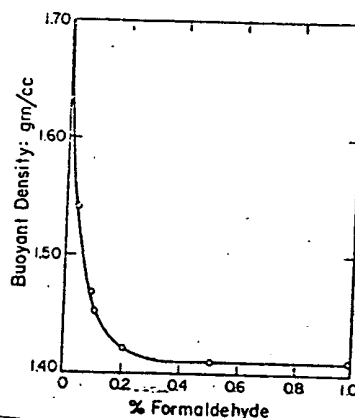


FIGURE 2: Buoyant density of nucleohistone complexes as a function of treatment formaldehyde concentration.

solved in 10 M urea. These samples were then electrophoresed on 15% polyacrylamide gels as previously described (Bonner *et al.*, 1968).

**Pronase Treatment.** Nucleohistone samples at a concentration of 1 OD<sub>260</sub> in 0.01 M triethanolamine (pH 7.8) were treated with pronase for 60 min at 37° by the addition of 20 μl/ml of sample of pronase solution (CalBiochem, grade B), 1 mg/ml, which had been predigested for 90 min at 37°. Sequential pronase treatment was done by adding further 20 μl/ml of sample of freshly predigested pronase 1 hr after the preceding treatment.

**DNase Treatment.** For treatment with DNase, the nucleohistone samples were 1 OD<sub>260</sub>, 0.01 M triethanolamine (pH 7.8), and 0.001 M MgCl<sub>2</sub>. These samples were cloudy before, during, and after the reaction. To the turbid solution was added 50 μl of DNase II (Worthington, electrophoretically pure), 1 mg/ml, and incubation at room temperature for 60 min.

## Results

When native pea bud nucleohistone is centrifuged to equilibrium in a CsCl density gradient, it yields a homogeneous band of DNA with a mean density of 1.695 g/cc (Figure 1A). The released protein forms a skin at the meniscus. Nucleohistone treated with concentrations of formaldehyde greater than 0.2% forms a complex whose buoyant density approaches 1.410 g/cc (Figure 2). Using the buoyant density of free histone (1.245 g/cc) in the density relation,  $V \cong 1/\rho; (1 + X)V_c = V_{DNA} + XV_{Histone}$ , where  $V_c$  is the  $V$  of the complex,  $V_{DNA}$  of the DNA,  $V_{Histone}$  of the histone, and  $X$  the weight ratio of histone to DNA in the complex; the ratio  $X$  for the complex can be calculated and is 1.27<sup>3</sup> in agreement with chemical analysis. The influence of formaldehyde on the density of the complex is therefore slight. Figure 2 shows the buoyant density of the complex as a function of the formaldehyde concentration during treatment. From these densities, the amount

<sup>2</sup> This relation ignores differences in hydration of histone and DNA due to variation in the activity of water at different CsCl concentrations and it ignores the difference between the buoyant density and the reciprocal of the partial specific volume. It is accurate enough for the present purpose. See Hearst and Vinograd (1961) and Meselson *et al.* (1957).

<sup>3</sup> Partial specific volume calculated from the amino acid composition of histone.

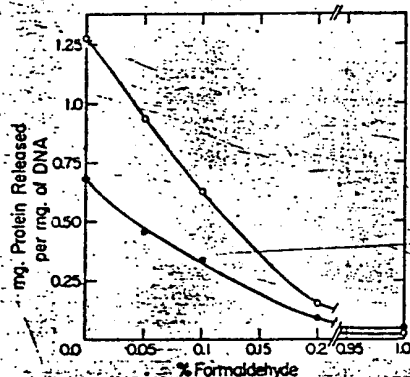


FIGURE 3: Amounts of protein bound in formaldehyde-treated complexes expressed as per cent of total protein present in the original nucleohistone. (A) Open circles: protein released as calculated from the buoyant density of the complex; (B) closed circles: amount of protein released by acid extraction as determined by chemical analysis. Scale  $2\times$  that of A.

of protein bound to DNA in a form resistant to dissociation by CsCl may be calculated (Figure 3). The amount of protein bound in acid-nonextractable form was determined by chemical analysis of nucleohistone treated with varied concentrations of formaldehyde. Interestingly, the proportion of protein not dissociable by CsCl is in close agreement with that not acid extractable. The protein dissociated in CsCl from nucleohistone was also banded in the density range 1.2–1.3 g/cc and was observed to decrease in quantity with increasing formaldehyde treatment concentration.

The buoyant band shapes of formaldehyde-treated nucleohistone are also a function of formaldehyde concentration (Figure 1). The band formed by pure DNA (Figure 1A) is very symmetrical and the band width indicates an apparent molecular weight<sup>4</sup> of  $2.0 \times 10^6$ . After treatment with 0.05% formaldehyde, 30% of the chromosomal protein remains bound to and bands with the DNA. The band is, however, broad and skewed (Figure 1B). Treatment with higher concentrations of formaldehyde causes sharpening of the peak and an increase in its symmetry as well as a decrease in its density (Figure 1C–F). Treatment with formaldehyde concentration higher than 1% causes the band to separate into two bands, the lighter having a buoyant density of 1.410 g/cc. Neither the band shape nor buoyant density of purified DNA is changed by treatment with 1% formaldehyde under the present conditions (Table I).

The sedimentation coefficient of nucleohistone is altered by formaldehyde treatment. Thus, the  $s_{20,w}$  of the original nucleohistone was 26 S; after treatment with 0.1% formaldehyde, 17 S; with 0.5% formaldehyde, 19 S; and with 1% formaldehyde, 21 S. In general the sedimenting bands of the formaldehyde-treated complex although single are broader than those of untreated nucleohistone.

Nucleohistone treated with formaldehyde shows both a

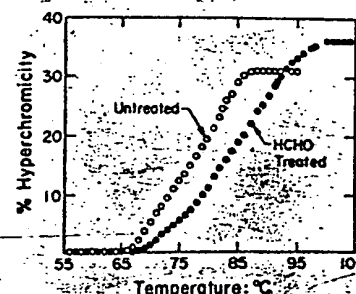


FIGURE 4: Normalized melting profiles of native nucleohistone and of nucleohistone treated with 1% formaldehyde.

greater thermal stability and a greater hyperchromicity upon melting than does the native material (Figure 4). The melting profile of treated nucleohistone is also broader. The increase in  $T_m$  as a function of formaldehyde concentration parallels the amount of protein bound in nondissociable form (Figure 5). Treatment of purified DNA with 1% formaldehyde as described above and after removing the formaldehyde by dialysis alters neither its  $T_m$  nor its hyperchromicity (Table I).

The protein which remains acid extractable from nucleohistone after formaldehyde treatment was subjected to examination by disc electrophoresis to determine the kinds of histone present (Figure 6). Treatment with even the lowest concentration of formaldehyde (0.05%) causes all of the lysine-rich histones Ia and Ib to become non-acid extractable. Treatment with 1% formaldehyde causes all but 3% of the protein of nucleohistone to become non-acid extractable. The 3% which is extractable is mainly histone II. There are thus substantial differences in reactivity with formaldehyde of the several species of histone.

The complex formed by reaction of nucleohistone with 1% formaldehyde was used as a substrate for DNase II or for pronase, and the digestion products banded in CsCl. The turbid solutions from the DNase reaction became clear immediately upon addition of CsCl. After incubation of nucleohistone with DNase II, no band of material formed in the density range 1.4–1.75 g/cc. Rather, the optically absorbing material remained dispersed throughout the entire CsCl gradient (Figure 7A). The DNA is thus degraded to small molecular weight pieces which are not held together by the proteins. Treatment with pronase (60 min) results in a complex of density 1.634 g/cc and of asymmetric band shape (Figure 7B).

TABLE I: Properties of DNA as a Function of Treatment with Formaldehyde.

	Purified Pea Bud DNA	1% Form- aldehyde- Treated DNA
Buoyant density	1.695	1.697
$T_m$ (deg)	45	45
Hyperchromicity (%)	38	38

<sup>4</sup> The apparent molecular weights differ from the expected values of about  $10^6$  for the DNA and the nucleohistone (based on sedimentation coefficients) because of band broadening due to minor heterogeneities in molecule weight and buoyant density (due to the G + C content).

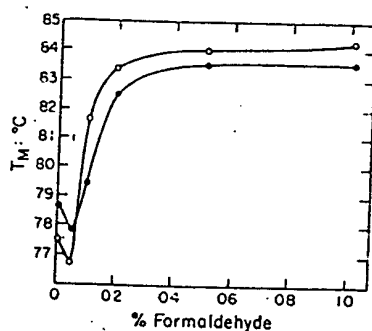


FIGURE 5: The  $T_m$  of treated nucleohistone as a function of treatment formaldehyde concentration. Each curve represents the results obtained from one preparation of nucleohistone.

Repeated (four times) pronase treatment of formaldehyde-treated complex resulted in material which banded at a density of 1.669 g/cc and from which therefore 96% of the protein had been removed (Figure 7C).

To determine the number and nature of the cross-links introduced, nucleohistone was treated in the standard manner with 1%  $^{14}\text{C}$ -labeled formaldehyde (sp act. 1 mCi/mmmole). After reaction, a portion was precipitated and washed with 10% trichloroacetic acid and counted to determine the total amount of formaldehyde which had reacted. A further portion was subjected to repeated pronase treatment as described above. The extensively pronased sample was next precipitated and washed with 10% trichloroacetic acid and then counted to determine the number of pronase-resistant cross-links. The results are given in Table II. The treated nucleohistone contained approximately 1 formaldehyde cross-link per 2 base pairs or per 15 amino acid residues. Pronase treatment removed 96% of the protein and also approximately two-thirds of the formaldehyde cross-links. These must then represent interprotein cross-links. The protein-DNA cross-links are represented by the formaldehyde which remains attached to the complex after pronase treatment. The ratio of amino acids to formaldehyde in the pronased material, 1.67, indicates the presence of 1-2 amino acids/cross-link. Pronase therefore removes all of the protein down to a stub of this length through which the protein was originally cross-linked to DNA.

#### Discussion

The treatment of nucleohistone with formaldehyde results in complexes in which protein-DNA interaction is no longer mainly ionic as it is in native nucleohistone. The treated complexes are dissociated neither by 4 M CsCl nor 0.2 N  $\text{H}_2\text{SO}_4$ . Reaction with formaldehyde results in other forms of DNA-protein interaction. Formaldehyde, used as a fixative, causes the formation of methylene bridges between two neighboring amino groups. The amino groups in purified DNA are not cross-linked under the conditions employed here. However, association of DNA with histone may encourage the formation of such bonds as well as of DNA-histone cross-links. The existence of the latter is indicated by the residual 4% bound protein which pronase treatment cannot remove and which bands with DNA in CsCl. This amount of protein corresponds to one amino acid for each four base pairs.

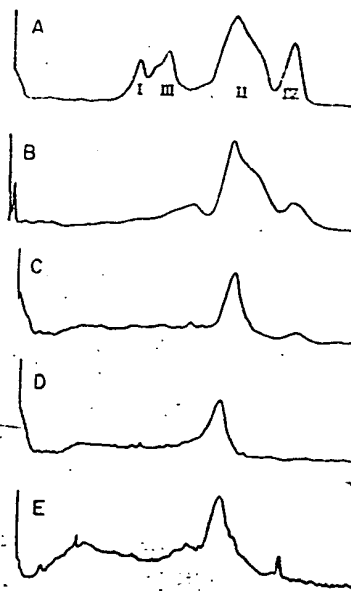


FIGURE 6: Microdensitometer traces showing disc gel electrophoresis patterns obtained from the acid-extractable proteins of formaldehyde-treated nucleohistone. (A) Untreated; (B) 0.05% formaldehyde; (C) 0.1% formaldehyde; (D) 0.5% formaldehyde; (E) 1.0% formaldehyde. Roman numerals refer to classes of histone: I, lysine rich; II, slightly lysine rich; III and IV, arginine rich.

Since the sedimentation coefficient does not change markedly, the buoyant band broadening caused by treatment with very low formaldehyde concentrations must be due to a distribution of histone/DNA ratios, the denser species being those containing less bound protein. Treatment with higher formaldehyde concentrations causes more of the total protein to be bound and the symmetry of the buoyant bands indicates that the complexes formed under these conditions are very homogeneous from molecule to molecule with respect to histone/DNA ratio. The apparent molecular weight<sup>4</sup> of the material in such bands is  $3.0 \times 10^6$ . Treatment with formaldehyde concentrations above 1% causes a new band of greater

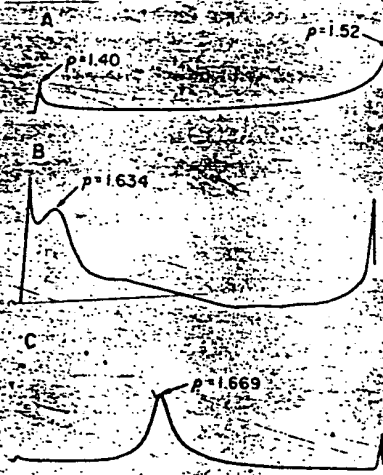


FIGURE 7: Buoyant density band patterns of the complex formed by the reaction of nucleohistone and 1% formaldehyde after treatment with (A) DNase II, (B) pronase, and (C) pronase four times.

TABLE II: Quantities of Formaldehyde Reacted with and Bound in Acid-Insoluble Form in Nucleohistone before and after Removal of Protein by Pronase.

Sample	Treatment	Buoyant Density	Protein Content (Amino Acid Base Pair)	Formaldehyde Reaction Product (Formaldehyde/ Base Pair)
Formaldehyde-treated nucleohistone	None	1.410	6.6	0.43
Formaldehyde-treated nucleohistone	Four-times-repeated pronase	1.669	0.25	0.15

density to appear. High concentrations of formaldehyde must therefore result in secondary alterations of nucleohistone structure or composition.

The increased  $T_m$  of formaldehyde-treated nucleohistone indicates that the DNA of the complex is more effectively stabilized by protein as a result of the more intimate binding between histone and DNA caused by the treatment. Such a stabilizing effect could result either from histone-histone methylene bridges or from histone-DNA bridges as described above.

The fact that histone I is completely missing from the acid-extractable proteins after treatment with very low concentrations of formaldehyde indicates that this histone is most susceptible of all histones in nucleohistone to attack by formaldehyde and is most readily bound to DNA. A portion of histone II is, on the contrary, acid extractable even after treatment with even the highest concentrations of formaldehyde. This histone must therefore be the least susceptible to attack by formaldehyde, indicating that it is more protected from solvent than are other histones of nucleohistone.

The fact that chromosomal proteins can, by treatment with formaldehyde, be joined to DNA and to each other in such a way that they do not dissociate from DNA in CsCl would seem to have many potential applications in chromosomal biology and chemistry. Thus, the method might be used to separate, on the basis of their buoyant densities, regions of DNA complexed with one class of protein from the DNA of other regions complexed with either other classes of proteins or with none at all.

#### Acknowledgment

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## A New Method of *in situ* Hybridization\*

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**Abstract.** A new method for gene mapping at the chromosome level using *in situ* hybridization and scanning electron microscopy is described and has been applied to mapping the rRNA genes of *Drosophila melanogaster*. Biotin is covalently attached to *Drosophila* rRNA via a cytochrome c bridge at a ratio of one cytochrome-biotin per 130 nucleotides by a chemical procedure. Polymethacrylate spheres with a diameter of ca. 60 nm are prepared by emulsion polymerization and are covalently attached to the protein avidin at a ratio of 5–20 avidins per sphere. The biotin-labeled rRNA is hybridized to denatured DNA in a chromosome squash. Upon incubation with a sphere solution, some of the biotin sites become labeled with spheres because of the strong non-covalent interaction between biotin and avidin. The chromosome squash is examined in the scanning electron microscope (SEM). Polymer spheres, which are visible in the SEM, are observed to label the nucleolus, where the rRNA genes are located.

### Introduction

The *in situ* hybridization technique was developed to map sites of specific genes and other interesting genetic sequences on chromosomes (Gall and Pardue, 1969; Pardue and Gall, 1969). The technique depends on the preparation of radio-labeled RNA or DNA probes complementary to sequences in the chromosome. These probes, which must be of high specific activity, and usually contain either <sup>3</sup>H or <sup>125</sup>I as the radioactive isotope, are hybridized to denatured DNA in a chromosome squash. The position of the complementary sequence in the chromosome is determined by light microscope autoradiography.

We wish to describe our initial studies of an alternative method of *in situ* hybridization which uses scanning electron microscopy and a non-radioactive label attached to the polynucleotide chain. For these initial tests we have chosen the 16S and 26S rRNA genes localized in the nucleolus and/or at the base of the X chromosome in the polytene chromosomes of *Drosophila melanogaster*. Further studies and developments are necessary before the sensitivity and resolution of this new method can be evaluated and compared to those of autoradiographic methods.

The basic features of our method are as follows.

(1) The labels are poly(methylmethacrylate) spheres with a diameter of ca. 60 nm. They are prepared by emulsion polymerization, chemically modified, and then covalently coupled to the protein avidin, so that there is a ratio of a few avidin molecules per sphere (avidin-spheres).

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(2) Biotin is covalently coupled to the RNA of interest (biotin-RNA) at a ratio of about one biotin per 100 to 200 nucleotides.

(3) Salivary gland chromosomes from isolated nuclei are spread on a glass slide and photographed in the light microscope (Mitchell and Lipps, 1975).

(4) The chromosomal DNA is denatured and hybridized with the biotin-RNA essentially as described by Pardue *et al.* (1970).

(5) The hybridized RNA-biotin is incubated with a suspension of avidin spheres and prepared for visualization in the scanning electron microscope (SEM).

The labeling reaction is based on the fact that avidin and biotin combine rapidly to form a remarkably stable complex (Green, 1963). Heitzmann and Richards (1974) have already described the use of the biotin-avidin interaction in electron microscope labeling procedures. We wish also to note that the use of polymer spheres for labeling studies has been described by Molday *et al.* (1974, 1975). Our studies on the use of the biotin-avidin reaction and of polymer spheres for nucleic acid labeling problems were initiated independently.

### Materials and Methods

**1. Preparation of Radioactive rRNA.** Ribosomal RNA containing a  $^3\text{H}$ -uridine label was obtained by microinjection of mid-third instar larvae with a dose of approximately one millicurie of  $^3\text{H}$ -uridine (70 Ci/mMole) per 250–300 larvae. After 4 hours incubation at 25°C the larvae were collected and washed with  $0.1 \times \text{SSC}$  (0.015 M NaCl, 0.0015 M Na citrate, pH 7.0). Polyribosomes were isolated and rRNA extracted by procedures described by Boschen (1970). The 26S and 16S rRNA components were further purified by velocity sedimentation through a 5 to 20% sucrose gradient in 0.1 M NaCl, 0.01 M acetate, pH 5.1, at 62 krpm for 2 hours at 4°C in the Spinco SW65 rotor. The 26S and 16S regions of the gradients were pooled and rRNA precipitated with two volumes of 80% ethanol. The rRNA was resuspended in 0.1 M NaCl, 0.01 M sodium acetate, pH 5.1, and stored at  $-20^\circ\text{C}$ .

**2. Preparation of rRNA-Cytochrome c-Biotin.** A cytochrome c bridge was used to attach biotin to the rRNA probe. The N-hydroxysuccinimidyl ester of biotin (NHS-B) was used to acylate some of the amino groups of cytochrome c, following the procedures of Becker *et al.* (1971) and Heitzmann and Richards (1974).

NHS-B was prepared as follows: A mixture of 3 millimoles of unlabeled biotin and 80  $\mu\text{l}$  of a 100  $\mu\text{Ci}/\text{ml}$  solution of  $^{14}\text{C}$ -biotin (50  $\mu\text{Ci}/\mu\text{mole}$ , Amersham/Searle) was evaporated to dryness and dissolved in 9 ml of dimethylformamide at 60°C. Three millimoles of N-hydroxysuccinimide were added and dissolved followed by 4 millimoles of dicyclohexylcarbodiimide. This solution was stirred at room temperature overnight. A white precipitate of the dicyclohexylurea formed in about 5–10 minutes. The solution and precipitate were separated and the solution reduced to dryness by rotary evaporation. The resulting solid was washed with several volumes of hot methanol. The remaining solid contained the bulk of the  $^{14}\text{C}$  count (~7000 cpm/mg). This precipitate, although probably not pure, contained the NHS-B in a form suitable for its subsequent use.

A 2.0  $\mu\text{l}$  solution containing 10 mg/ml of cytochrome c in 0.1 M  $\text{NaHCO}_3$  was mixed with 0.2 ml of dimethylformamide containing 1.6 mg (~4.7  $\mu\text{moles}$ ) of the radioactive NHS-B. The mixture was incubated at room temperature for 1 hour, passed over a 15  $\times$  2 cm Sephadex G-25 column and eluted with 0.15 M NaCl. The cytochrome c fraction was collected and stored at 4°C. 100% of the input biotin was covalently attached to the protein (3 biotin per cytochrome c).

Biotin-labeled cytochrome c was coupled to rRNA by reaction with formaldehyde using a modification of the procedure of Brutlag *et al.* (1969). 0.05 ml of a cytochrome c-biotin solution (3 mg/ml) was added to 1.0 ml of an rRNA solution (100  $\mu\text{g}/\text{ml}$ ) after each had been dialyzed against 0.01 M triethanolamine (TEA), pH 7.8. 0.11 ml of 6% formaldehyde in the



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reaction with formaldehyde using 0.05 ml of a cytochrome c-biotin (100  $\mu\text{g}/\text{ml}$ ) after each had been 11 ml of 6% formaldehyde in the

ame buffer was added, and the mixture incubated at  $37^\circ\text{C}$  for 30 minutes. Formaldehyde was removed by dialysis against four or more changes of TEA buffer at  $4^\circ\text{C}$  for 48 hours; 5.0 M NaCl was then added to give a final concentration of 1.0 M. This solution was fractionated on a  $28 \times 1.3$  cm column of Sepharose 6B in 1.0 M NaCl, 0.001 M EDTA, pH 8.0. The elution of the RNA and of the cytochrome c was detected by absorbance at 260 nm and 410 nm, respectively. Extinction coefficients of  $25 \text{ cm}^2/\text{mg}$  for the RNA and  $7.7 \text{ cm}^2/\text{mg}$  for the cytochrome c were used to calculate a nucleotide to cytochrome ratio of 130:1 in the product. The biotin-rRNA was dialyzed against  $2 \times \text{SSC}$ , pH 7.5. For storage, the sample was divided into small fractions and frozen at  $-70^\circ\text{C}$ .

It should be noted that this procedure relies on the fact that the positively charged protein, cytochrome c ( $\text{pI} \approx 10.5$ ), binds by electrostatic interactions to RNA at low ionic strength (0.1 M) thus favoring the formaldehyde crosslinking reaction. Any cytochrome c which is not covalently bound to the nucleic acid is dissociated at the high ionic strength (1.0 M) used in the Sepharose column fractionation (Olivera, 1966).

**3. Preparation and Properties of Polymer Spheres.** Polymer spheres were prepared by emulsion polymerization. Methyl methacrylate (2.25 g, Polysciences), ethylene dimethacrylate (0.10 g, Polysciences), methacrylic acid (0.15 g, Polysciences), water (47 ml, redistilled) and sodium dodecyl sulfate (0.12 g, MC&B, USP grade) were measured into a 100 ml round bottom flask and mixed thoroughly. The mixture was sonicated for a few seconds (power 4, Branson Sonifier Model S-125) to produce a white emulsion. Argon was bubbled through the emulsion for several minutes. A solution of 60 mg of  $\text{K}_2\text{S}_2\text{O}_8$  in 10 ml water was then added to the stirred emulsion, and deoxygenation with argon was repeated. The flask was stoppered with a rubber septum and heated at  $60^\circ\text{C}$  with stirring for 38 hours. The emulsion was stored at  $4^\circ$  in 1 mM EDTA.

The emulsion was treated with NaOH in order to hydrolyze some of the ester groups thus increasing the surface concentration of carboxylate anions and probably displacing some of the SDS from the sphere surface. Equal volumes of a sphere emulsion, as described above, and 2 M NaOH were mixed and heated at  $85^\circ\text{C}$  for 100 hours. The emulsion was dialyzed exhaustively at  $4^\circ$  against 0.10 M NaCl, 0.01 M sodium phosphate, 0.001 M EDTA (pH 7.0), then against water, and finally against 1 mM EDTA. The resulting sphere emulsion was stored at  $4^\circ\text{C}$ .

Sphere concentrations were estimated by weighing the residue left on evaporation of a weighed aliquot of emulsion which had been exhaustively dialyzed against water. In order to measure the diameter of the spheres and to observe their general morphology, they were mounted for electron microscopy from a sphere concentration of 40–50  $\mu\text{g}/\text{ml}$  by the formamide modification of the Kleinschmidt cytochrome c procedure. The hyperphase was 50% formamide, 0.10 M Tris, 0.01 M EDTA (pH, 8.5) and the hypophase was 17% formamide, 0.01 M Tris, 0.001 M EDTA (pH 8.5). The film was rotary shadowed with Pt-Pd. A typical transmission electron micrograph (Fig. 1a) shows the spheres to be approximately round and well dispersed. The measured diameters gave a rather narrow symmetrical distribution curve with a peak at 58 nm and with 67% of the values falling within the range 45–62 nm. Assuming a density of  $1.24 \text{ g}/\text{ml}$ , the average molecular weight of a sphere with a diameter of 58 nm is calculated to be  $7.6 \times 10^7$  daltons. Fig. 1b illustrates the appearance of the spheres as photographed in the scanning electron microscope at high magnification. A solution containing 0.5  $\mu\text{g}$  of spheres in 5 ml of cesium chloride solution of average density  $1.20 \text{ g}/\text{ml}$  was centrifuged for 40 hours at 100000 g. The position of the sphere band was readily observed from the turbidity and corresponded to a density of  $1.27 \text{ g}/\text{ml}$ . Spheres banded in sodium iohalamate solution (Serwer, 1975) at a density of 1.226.

**4. Covalent Coupling of Avidin to Spheres.** Avidin-labeled polymethacrylate spheres (avidin-spheres) were prepared by coupling avidin (Sigma) and spheres with 1-cyclohexyl-3-(2-morpholinyl)-(4-ethyl)-carbodiimide methyl p-toluene sulfonate (Aldrich).

Spheres at a concentration of 21  $\text{mg}/\text{ml}$  were suspended in 1.0 mM EDTA. Then 0.72 ml of 0.05 M carbodiimide solution was added to 3.6 ml of the sphere suspension, followed by 1.44 ml of avidin solution (2  $\text{mg}/\text{ml}$ ). The mixture was incubated at  $25^\circ\text{C}$  for 16 hours and the reaction was quenched by addition of 0.01 ml of neat 2-ethanolamine. 5.0 M NaCl was added to a final concentration of 1.0 M. The mixture was divided into 1.0 ml aliquots and layered onto a 3.0 ml, 5 to 20% sucrose gradient over a 1.0 ml cushion of 60% sucrose, 1.0 M



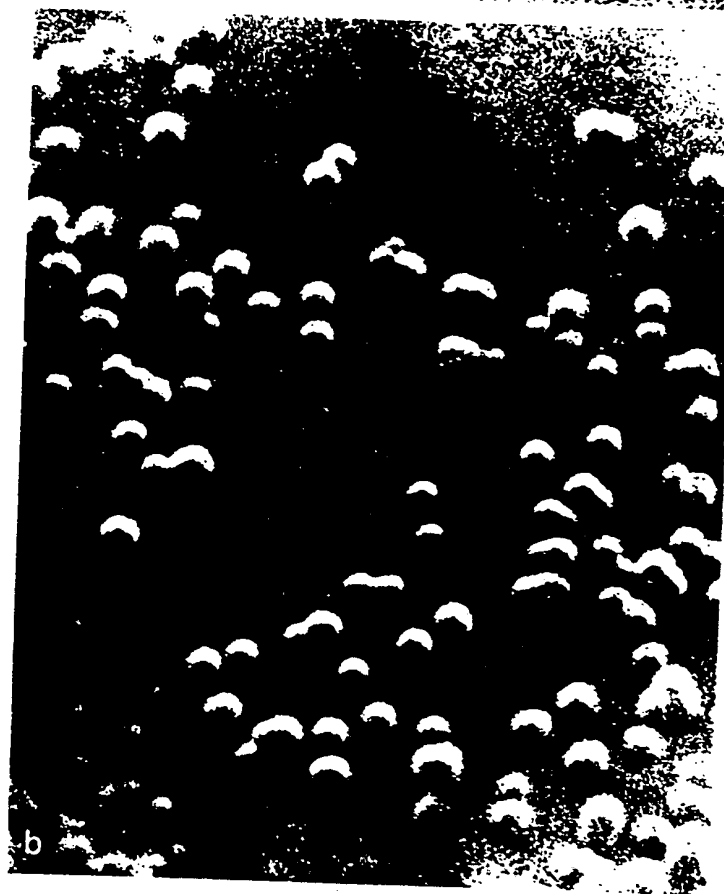


Fig. 1a and b

NaCl, 0.01 M EDTA, 35 krpm at 15 sphere band wa 0.01 M EDTA, 1.0 M NaCl, 0.0

Part of the charged protein but dissociated.

The number with  $^{14}\text{C}$ -biotin, chromatography prepared as des biotins per mol millimoles of bi are inactivated avidins may rea

5. Chromoso: isolated nuclei w on slides were fi the slides after f dried. The proced The slides were r tion, washed twi was carried out for 16-20 hours a of a solution of a over the chromos  $2 \times \text{SSC}$ , 70% and ing the chromoso coated with carbo

In *in situ* hy *Drosophila hyde* biochemical and located in or ne which maps at t interest in the p

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Fig. 1a and b. Tran was attached to ca the text for rRNA. was incubated with modification of the The spheres are ver have an average d (b) High magnifi

NaCl, 0.01 M EDTA, pH 8.5. After centrifugation for 1 hour in the Spinco SW50.1 rotor at 35 krpm at 15°C, the avidin-spheres had migrated to the top of the sucrose cushion. The sphere band was removed by dripping and dialyzed for 16 hours at 25°C against 1.0 M NaCl, 0.01 M EDTA, pH 8.5. The spheres were again centrifuged as above and finally stored in 1.0 M NaCl, 0.01 M EDTA, pH 8.5 at 4°C.

Part of the rationale for this preparative and separation procedure is that the positively charged protein, avidin, is electrostatically bound to spheres at low electrolyte concentration but dissociates, unless covalently coupled, at high electrolyte concentration.

The number of biotin binding sites per sphere was assayed by testing the avidin spheres with  $^{14}\text{C}$ -biotin, followed either by repeated dialysis to remove unbound biotin or by exclusion chromatography through Sepharose 6B. The number of biotin binding sites per avidin-sphere, prepared as described, was 19. Pure avidin, with a molecular weight of 66000, binds four biotins per molecule (Green and Toms, 1970). Our starting preparation bound about 3.2 millimoles of biotin per 66000 mg of protein. It is not known how many of the binding sites are inactivated when avidin is covalently linked to the spheres. Thus, the number of bound avidins may reasonably be estimated to lie between 5 and 19.

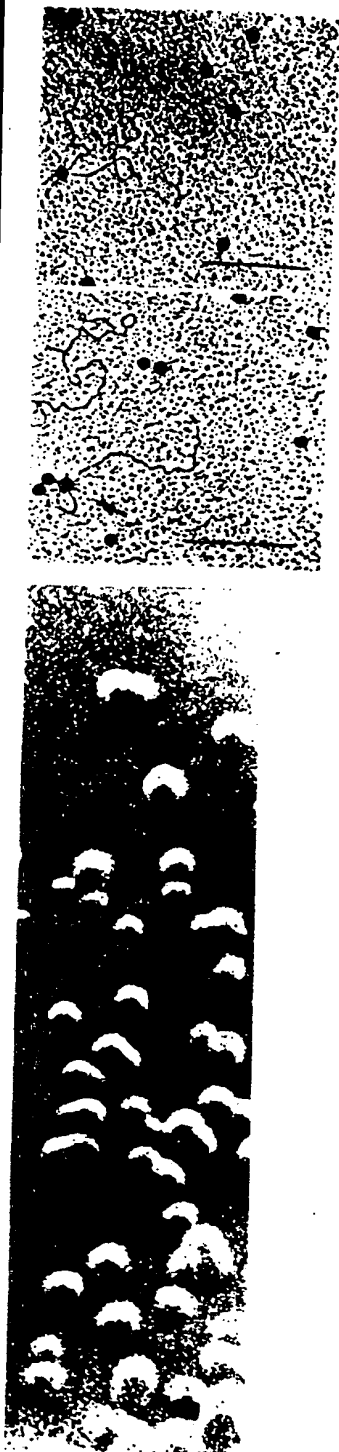
5. *Chromosome Preparation and in situ Hybridization.* Chromosome preparations from isolated nuclei were made essentially as described by Mitchell and Lipps (1975). Chromosomes on slides were first photographed under phase contrast. The cover slips were removed from the slides after freezing in liquid nitrogen. The slides were rinsed in 100% ethanol and air dried. The procedure for *in situ* hybridization is essentially as described by Pardue *et al.* (1970). The slides were rinsed in  $2\times$  SSC, incubated in 0.07 M NaOH for 2 minutes to effect denaturation, washed twice in 70% ethanol and twice in 95% ethanol before drying. Hybridization was carried out at a concentration of rRNA-biotin of 2  $\mu\text{g}/\text{ml}$  in  $2\times$  SSC, 50% formamide for 16–20 hours at 25°C (Alonso, 1973). The slides were rinsed four times in  $2\times$  SSC. 300  $\mu\text{l}$  of a solution of avidin-spheres (40  $\mu\text{g}/\text{ml}$  in 1.0 M NaCl, 1 mg/ml cytochrome c) was placed over the chromosomes. The slides were maintained at 25°C for 4 hours, extensively rinsed in  $2\times$  SSC, 70% and 95% ethanol and then allowed to air dry. That portion of the slide containing the chromosome material was cut to a 2 cm square, mounted on an aluminum stud, and coated with carbon and/or gold for examination in the SEM.

## Results

In *in situ* hybridization experiments Pardue *et al.* (1970) found the rDNA of *Drosophila hydei* solely within the nucleolus. Their results are consistent with biochemical and genetic studies on *D. melanogaster* that show the genes to be located in or near the nucleolus organizer region (Ritossa and Spiegelman, 1965), which maps at the base of the X chromosome. Therefore the region of particular interest in the present study is the nucleolus.

Chromosomes mounted directly for SEM by the methods of Mitchell and Lipps (1975) have very clear morphological features including a banding pattern which correlates well with the pattern seen by light microscopy. However, treatment with NaOH, which is necessary for *in situ* hybridization, causes flattening of the chromosomes and loss of many of the recognizable morphological features.

Fig. 1a and b. Transmission and scanning electron micrographs of polymer spheres. (a) Biotin was attached to calf-thymus DNA via a cytochrome c bridge by the procedures described in the text for rRNA, at a ratio of one cytochrome c-biotin per 700 nucleotides. This solution was incubated with avidin-spheres, and mounted for electron microscopy by the formamide modification of the standard Kleinschmidt procedure (Davis, Simon, and Davidson, 1970). The spheres are very opaque because the grids were rotary shadowed with Pt-Pd. The spheres have an average diameter of 58 nm; the calibration bar is 1,000 nucleotides or 0.32  $\mu\text{m}$ . (b) High magnification SEM picture of spheres on a chromosome squash. ca  $\times 100,000$



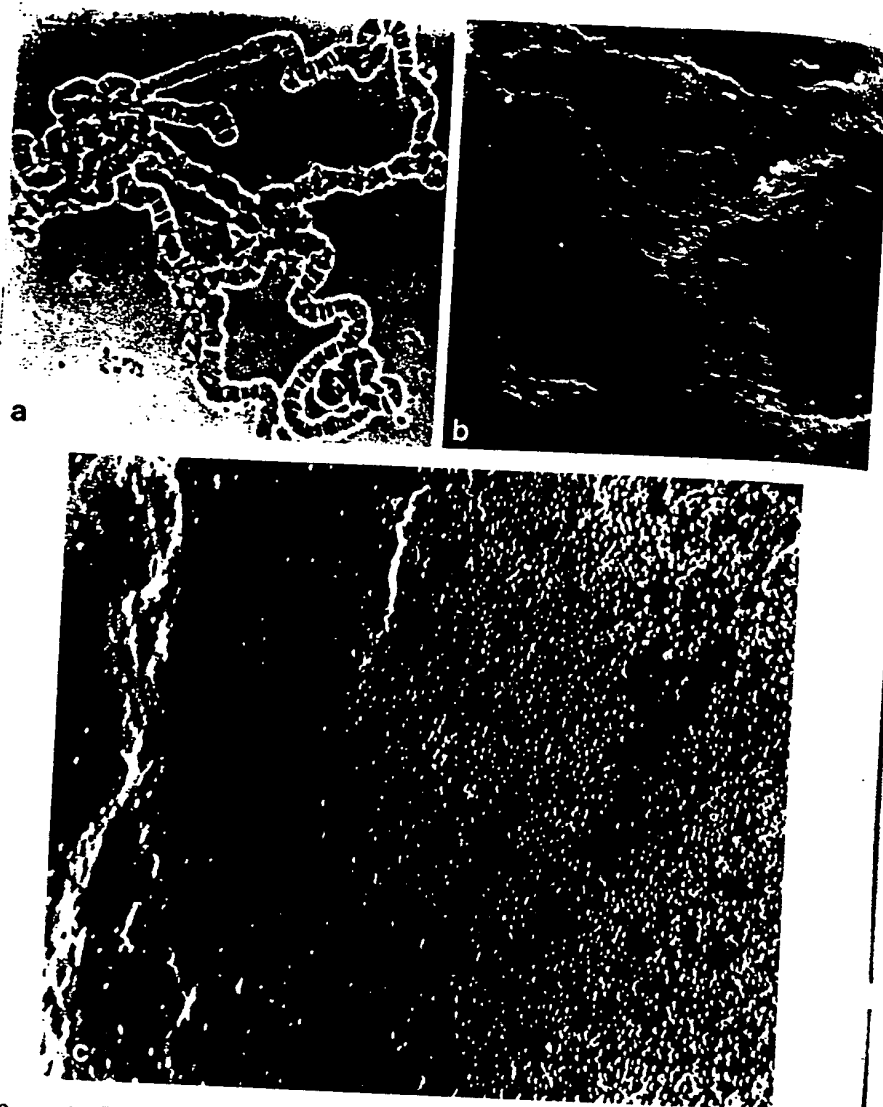


Fig. 2a—c. (a) *Drosophila* chromosomes photographed by phase optics in the light microscope prior to denaturation and hybridization. (b) Low magnification SEM picture of the region around the chromocenter and nucleolus after *in situ* hybridization. Bars in (a) and (b) represent  $10\ \mu\text{m}$ . (c) Higher magnification SEM picture of the region including part of the X chromosome and part of the nucleolus, as indicated in the square and parallelogram in the top pictures. The nucleolus is densely labeled with spheres. ca.  $\times 10,000$

The general silhouette of the chromosomes as viewed from above is, however, still clearly recognizable. Positions on the chromosomes can be identified by correlation of the SEM pattern with the photomicrograph obtained under phase optics prior to the NaOH treatment.



Fig. 3a and b. (a) Li prior to denaturation tip of the X chromosome (b) after *in situ* SEM picture (Fig.

Fig. 2 shows a micrograph of the chromocenter and SEM picture (Fig.

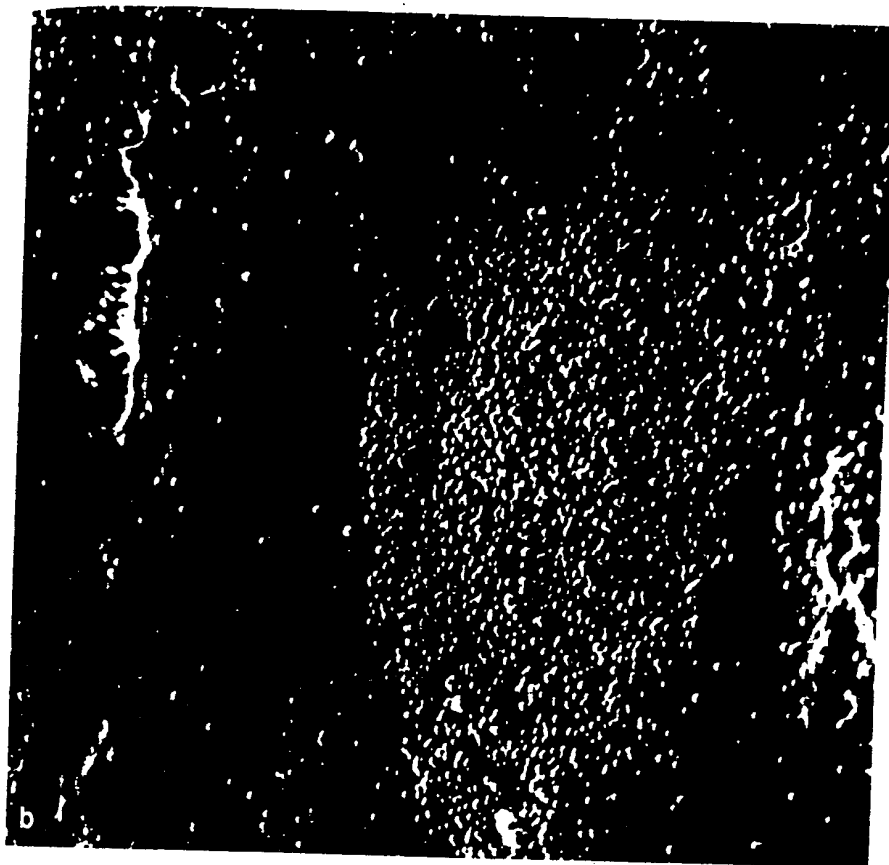


Fig. 3a and b. (a) Light micrograph of a detached X chromosome with a nucleolus at its base, prior to denaturation and hybridization. Bar represents 10  $\mu$ m. (b) SEM picture of part of the tip of the X chromosome and the nucleolus (the region within the square in the light micrograph) after *in situ* hybridization, showing a dense labeling of the nucleolus with spheres. ca.  $\times 15,000$

Fig. 2 shows the results of a typical hybridization experiment. The light micrograph of the chromosomes (Fig. 2a) shows that the nucleolus occurs at the chromocenter and close to the base of the X chromosome. In a low magnification SEM picture (Fig. 2b) the general morphology of the chromosomes in this region

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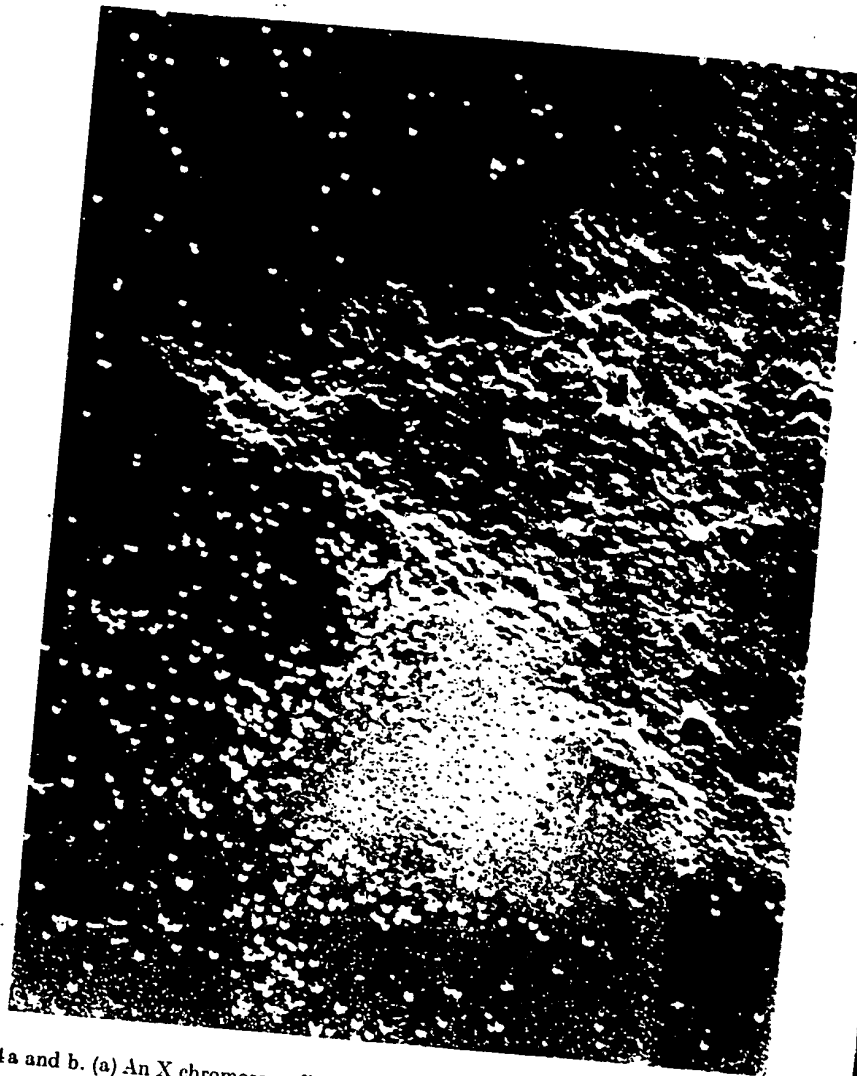


Fig. 4a and b. (a) An X chromosome fixed in 1:1 acetic:lactic acid, with no visible nucleolus. Bar represents 10  $\mu$ m. (b) As shown in the SEM, after *in situ* hybridization, there is a low, but above background, density of spheres at the tip of the X chromosome. ca.  $\times 15,000$

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is apparent. At this magnification the polymer spheres are visible as small dots. A high density of spheres covers the nucleolus, while a much lower sphere density over other regions of the chromosome and over the rest of the slide is apparent. The spheres may be seen more clearly in the moderately high magnification SEM picture of a small section of the nucleolus (Fig. 1c). There are very few spheres over the adjoining regions of the X chromosome. The chromosome arms and the contaminating cytoplasmic material were carefully examined; no other position with a significant number of spheres was observed.

Fig. 3 shows another typical result. In this case, the pressure applied during chromosome squashing has resulted in the detachment of the X chromosome from the chromocenter. A portion of the nucleolus remains attached to the base of the X chromosome (Fig. 3a). The high magnification SEM picture of this region (Fig. 3b), after hybridization and labeling, shows a very high density of spheres over the nucleolus.

In chromosome preparations treated with 45% acetic acid (such as shown in Figs. 2 and 3) the nucleolar material is readily observed under phase microscopy. However, in preparations fixed in a 1:1 acetic:lactic acid mixture, the nucleolus is no longer observable. In such preparations, as illustrated in Fig. 4, we found that the spheres, usually in relatively small numbers, were localized near the heterochromatin material at the base of the X chromosome. This suggests the presence of rDNA radiating out from this region.

Control experiments were performed in which the hybridization was carried out with rRNA which was not coupled to biotin. These preparations showed no localization of spheres over the nucleolus or any other part of the chromosomes.

As described in Methods (p. 000), the incubation with avidin-spheres is carried out in the presence of 1 mg/ml of cytochrome c. If cytochrome c is not used, there is a high level of nonspecific attachment of spheres all over the slides. Avidin is a very positive protein with an isoelectric point of ca. 10.5 (Melamed and Green, 1963). Presumably the positively charged cytochrome c competes for negatively charged sites on the slides and thus decreases the amount of nonspecific labeling.

In the procedure described, we observed no localization of spheres on any region of the chromosome other than that containing nucleolar material. But we have observed on some slides that there are areas which contain no chromosome structures but are heavily labeled with spheres. These dense areas were observed using both rRNA-biotin and rRNA without biotin. The cause of this occasional dense labeling is not known. However, its occurrence is infrequent and does not pose a significant problem for the identification of reiterated genes such as the ribosomal genes.

### Discussion

In developing a sphere labeling procedure, we have been guided by the philosophy that the polynucleotide probe (in this case rRNA) should be only mildly modified, so that steric hindrance in the slow hybridization reaction is minimal. The massive sphere label then attaches by a reaction between biotin and avidin, which is rapid and complete for the free molecules.

The results reported here show that the polymer sphere labeling procedure and SEM observation is effective for mapping the 200-fold reiterated (Tartof

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and Perry, 1970) rRNA genes in the polytene chromosomes of *Drosophila melanogaster*. In principle, the method should give higher resolution than autoradiography at the optical microscope level. It is in some ways more convenient than autoradiography because long exposure times are avoided. It can be applied to any polynucleotide and does not require high specific activity radioactive labeling.

The DNA in the nucleolus appears to be well dispersed and may be especially readily available for hybridization and for the subsequent penetration and reaction of avidin-spheres. Further tests will be carried out to determine the effectiveness of the procedure for reiterated genes occurring in other regions of the chromosome, for single-copy genes in polytene chromosomes, and for gene mapping in nonpolytene chromosomes.

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*Drosophila melanogaster* than autoradiography is more convenient than can be applied to radioactive labeling. It may be especially useful for determination and re-determination of the efficiency of her regions of the gene map.

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# Ribonucleic Acid-Protein Cross-Linking in *Escherichia coli* Ribosomes: (4-Azidophenyl)glyoxal, a Novel Heterobifunctional Reagent<sup>†</sup>

Samuel M. Politz, Harry F. Noller,\* and Paul D. McWhirter

**ABSTRACT:** We have used the heterobifunctional reagent (4-azidophenyl)glyoxal (APG) to cross-link RNA to protein in *Escherichia coli* 30S ribosomal subunits. Synthesis and characterization of the reagent are described. Like other dicarbonyl reagents (e.g., kethoxal), APG reacts specifically with guanosine among the four ribonucleosides. The azido group in APG can be photolyzed with UV light ( $\lambda > 300$  nm),

yielding an unstable nitrene which is potentially reactive with many groups in proteins and nucleic acids. Conditions for APG modification of guanylic acid residues in 30S subunits are described; photolysis of bound APG results in cross-linking of approximately 5% of the total 30S proteins to 16S RNA. A specific subset of the 30S proteins is cross-linked to 16S RNA by APG.

**R**ibosomes are the largest and most complex components of the cellular machinery for protein synthesis. For example, the *E. coli* ribosome is composed of 52 different protein molecules and three RNA molecules, organized into two structurally and functionally distinct subunits. Determining the three-dimensional structure of the ribosome is a prerequisite for complete understanding of the mechanism of protein synthesis, but the complexity and asymmetry of the structure present major barriers to its solution.

Nevertheless, major advances have been made by using newly developed approaches. There is now a satisfying level of agreement on the detailed locations of some of the ribosomal proteins in a three-dimensional model of the ribosome (Moore, 1980; Lake, 1980). In contrast, detailed information exists for only one site in ribosomal RNA, the 3' terminus of 16S RNA, which has been localized to the same region of the *E. coli* 30S ribosomal subunit in four independent studies (Poltz & Glitz, 1977; Olson & Glitz, 1979; Shatsky et al., 1979; Stöffler et al., 1980). Possible locations of other domains in ribosomal RNA have been proposed, but the evidence for these inferences has been of necessity indirect (Zimmerman, 1980; Bogdanov et al., 1980; Noller, 1980).

The need for direct examination of the structural disposition of ribosomal RNA in the ribosome has led to the development of new methods for determining the physical proximity of specific ribosomal proteins to specific sites in ribosomal RNA. Principal among these new approaches has been the exploitation of photochemical and chemical cross-linking of ribosomal proteins to ribosomal RNA [reviewed in Zimmermann (1980)]. Either by direct UV<sup>†</sup> irradiation of ribosomes or by use of bifunctional cross-linking reagents, several groups have succeeded in cross-linking proteins to ribosomal RNA. In only one case, however, has the subsequent analysis of cross-linked products been carried through to unambiguously identify the specific cross-linked peptide and oligonucleotide (Möller et al., 1978; Zwieb & Brimacombe, 1979).

Direct UV irradiation of ribosomes at low doses cross-links only three specific proteins to ribosomal RNA (Möller & Brimacombe, 1975; Baca & Bodley, 1976; Möller et al., 1978). Higher doses cause such extensive cross-linking that cooper-

ative unfolding of subunits appears to occur (Möller & Brimacombe, 1975; Gorelic, 1976). Bifunctional chemical reagents for cross-linking have been less extensively exploited, largely due to the scarcity of suitable reagents (Zimmermann, 1980; Ulmer et al., 1978). We have developed a bifunctional reagent, (4-azidophenyl)glyoxal (APG), which has potential for investigation of RNA-protein topography in the ribosome. Like other dicarbonyl reagents (e.g., kethoxal), APG should react with guanosine (Staehelin, 1959; Shapiro & Hachmann, 1966; Litt & Hancock, 1967; Noller, 1974) at sites in RNA which are not involved in base-pairing interactions (Litt & Hancock, 1967; Litt, 1969; Noller, 1974). Thus, a relatively small number of sites in ribosomal RNA should be modified, minimizing effects of the reagent on ribosome conformation. Once APG is covalently bound to ribosomes via the dicarbonyl group, the azide functional group can be selectively photolyzed with long wavelength ultraviolet light, generating an unstable nitrene which is potentially reactive with many chemical groups in both proteins and nucleic acids (Bayley & Knowles, 1977). Since cross-linking is a two-step reaction, unbound APG can be removed before photolysis, thereby minimizing the cooperative unfolding and aggregation problems encountered with other reagents (Baumert et al., 1978; Ulmer et al., 1978). Furthermore, the extensive information obtained in this laboratory on sites of kethoxal modification in *E. coli* 16S and 23S ribosomal RNA should facilitate analysis of the cross-linked RNA (Noller, 1974; Chapman & Noller, 1977; Hogan & Noller, 1978; Herr & Noller, 1978).

We describe here the synthesis and characterization of APG, its reaction with guanosine, RNA, and ribosomes, and its properties in cross-linking protein to RNA in the *E. coli* 30S subunit. We also report the preliminary characterization of several proteins cross-linked to 16S RNA by APG treatment of 30S subunits.

## Experimental Section

**Analytical Methods.** Ultraviolet spectra were taken on a Cary 14 spectrophotometer and infrared spectra on a Perkin-Elmer 237B instrument. Nuclear magnetic resonance

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<sup>†</sup> Abbreviations used: APG, (4-azidophenyl)glyoxal; UV, ultraviolet; butyl-BPD, 2-(4-*tert*-butylphenyl)-5-(4-biphenyl)-1,3,4-dioxazole; Me<sub>2</sub>SO, dimethyl sulfoxide; TLC, thin-layer chromatography; NaDodSO<sub>4</sub>, sodium dodecyl sulfate; EDTA, (ethylenedinitrilo)tetraacetic acid, disodium salt; NMR, nuclear magnetic resonance; PPO, 2,5-diphenyloxazole.

(NMR) spectra were obtained on a JEOL JNM-FX60 instrument. Microanalyses were performed on a Perkin-Elmer Model 240B elemental analyzer.  $^{32}\text{P}$  radioactivity in non-aqueous samples was measured in 10 mL of a cocktail containing 8.4 g of butyl-BPD (Beckman) per liter of toluene.  $^{35}\text{S}$  radioactivity in aqueous samples was measured in 10 mL of the same cocktail plus 10% (v/v) Biosolv (Beckman). A Beckman LS-200B liquid scintillation system was used.

**Synthesis of (4-Azidophenyl)glyoxal.** A 1.5-g portion of selenium dioxide (Matheson, Coleman and Bell) was dissolved in 7.3 mL of dioxane plus 0.25 mL of water by heating to 80 °C. A 2.0-g portion of 4-azidoacetophenone (Pochinok & Kalashnikova, 1954; Hepher & Wagner, 1960) was dissolved in 1 mL of dioxane and then added dropwise to the  $\text{SeO}_2$  solution. The reaction mixture was refluxed (100–110 °C) with stirring for 3.5 h and then filtered (Whatman no. 1) to remove solid selenium. (Note: Selenium and its compounds are toxic. Contact with skin was avoided; solid and liquid wastes contaminated with selenium were disposed of by institutional Environmental Health and Safety personnel.) After concentration of the filtrate in vacuo, the resulting oil was heated in 5 volumes of boiling water until no more would dissolve. A residual immiscible oil was removed from the bottom of the flask. The product, (4-azidophenyl)glyoxal monohydrate, crystallized from the aqueous solution after standing 48 h at 20 °C (600 mg, 25%). Recrystallization from benzene gave a melting point of 96–98 °C. The  $^1\text{H}$  NMR data were the following: ( $\text{Me}_2\text{SO}-d_6$ )  $\delta$  5.63 [br t, 1,  $\text{CH}(\text{OH})_2$ ], 6.74 [d, 2,  $\text{CH}(\text{OH})_2$ ], 7.23 (d, 2, aromatic), 8.13 (d, 2, aromatic); ( $\text{Me}_2\text{SO}-d_6$  plus 1 drop of  $\text{D}_2\text{O}$ )  $\delta$  5.63 [s, 1,  $\text{CH}(\text{OD})_2$ ], 7.23 (d, 2, aromatic), 8.13 (d, 2, aromatic). Other spectral data were the following: IR 2131 ( $\text{N}_3$ ), 1695 ( $\text{CO}$ )  $\text{cm}^{-1}$ ; UV (0.2% ethanol in  $\text{H}_2\text{O}$ )  $\lambda_{\text{max}}$  296 nm ( $\epsilon_{\text{max}}$   $2.33 \times 10^4$ ). Thin-layer chromatography of (4-azidophenyl)glyoxal hydrate [silica gel G, diethyl ether–hexanes (1:1)] gave an  $R_f$  of 0.40, compared to 0.88 for 4-azidoacetophenone. Anal. Calcd for  $\text{C}_8\text{O}_2\text{N}_3\text{H}_5\cdot\text{H}_2\text{O}$ : C, 49.74; H, 3.63; N, 21.76. Found: C, 49.06; H, 3.59; N, 21.97.

**Reaction of APG with Nucleosides and Nucleotides.** Guanosine was reacted for 2 h at 37 °C with kethoxal or APG in 100  $\mu\text{L}$  of solution containing 45% aqueous methanol, 0.1  $\mu\text{mol}$  of guanosine, and 0.9  $\mu\text{mol}$  of APG or 2.6  $\mu\text{mol}$  of kethoxal. Aliquots of 25  $\mu\text{L}$  of reaction mixtures were chromatographed on cellulose thin-layer sheets with 2-propanol–water (7:3) as eluant. Guanosine and reaction products were detected by viewing the chromatogram under ultraviolet light.

Mononucleotides obtained from alkaline hydrolysis of  $^{32}\text{P}$ -labeled *E. coli* 16S ribosomal RNA (specific activity approximately 10000 cpm/ $\mu\text{g}$ ) were separated by paper electrophoresis, eluted, and concentrated by lyophilization (Barrell, 1971) before reaction with APG. Reaction mixtures contained 1500 cpm of  $^{32}\text{P}$ -labeled UMP, GMP, AMP, or CMP and 0.1  $\mu\text{mol}$  of APG (0  $\mu\text{mol}$  of APG in controls) in 15  $\mu\text{L}$  of buffer F. Reaction mixtures were incubated for 1 h at 37 °C and then electrophoresed at pH 3.5 for 1.5 h at 3000 V (Barrell, 1971) on Whatman 3MM paper. Following autoradiography, spots were cut out from the paper and counted.

**Solution Properties of APG.** APG was routinely kept at –20 °C as a 200 mg/mL dioxane solution. Before use for chemical modification, the stock dioxane solution was diluted to an APG concentration of 6 mg/mL in buffer E. APG immediately precipitated but was redissolved by heating 5 min at 65 °C. After brief centrifugation to remove remaining traces of precipitate, the appropriate volume of APG solution was added to each sample containing ribosomal subunits. APG

did not reprecipitate after cooling to room temperature for at least 30 min.

**Buffers.** Buffer A: 0.1 M  $\text{NH}_4\text{Cl}$ , 0.01 M  $\text{MgCl}_2$ , 0.01 M Tris (pH 7.5), 1 mM EDTA, and 6 mM 2-mercaptoethanol. Buffer B: 0.5 M  $\text{NH}_4\text{Cl}$ , 0.01 M  $\text{MgCl}_2$ , 0.02 M Tris (pH 7.5), 0.5 mM EDTA, and 6 mM 2-mercaptoethanol. Buffer C: 0.03 M  $\text{NH}_4\text{Cl}$ , 1 mM  $\text{MgCl}_2$ , 0.01 M Tris (pH 7.5), and 6 mM 2-mercaptoethanol. Buffer D: 0.03 M  $\text{NH}_4\text{Cl}$ , 10 mM  $\text{MgCl}_2$ , 0.02 M Tris (pH 7.5), and 6 mM 2-mercaptoethanol. Buffer E: 0.1 M sodium cacodylate (pH 7.0), 0.02 M sodium borate, and 0.01 M  $\text{MgCl}_2$ . Buffer F: 0.1 M sodium cacodylate (pH 7.0), 0.02 M sodium borate, and 1 mM EDTA. Buffer G: 0.1 M sodium acetate (pH 6.0), 0.02 M sodium borate, 0.1 M LiCl, 1 mM EDTA, and 0.5% NaDodSO<sub>4</sub>. Buffer H: 0.1 M sodium acetate (pH 6.0), 0.02 M sodium borate, 0.1 M LiCl, 1 mM EDTA, and 0.1% NaDodSO<sub>4</sub>.

**Enzymes.** Ribonuclease T1 (Sankyo) was obtained from Calbiochem, ribonuclease A from Worthington, bacterial alkaline phosphatase from Sigma, and ribonuclease T2 (Sankyo) from Calbiochem. Standard enzyme solutions were prepared according to Barrell (1971).

**Preparation of Ribosomal Subunits and Proteins.** For  $^{32}\text{P}$ -labeled ribosomes, *E. coli* MRE600 cells were grown on low-phosphate medium (Garen & Levinthal, 1960) as described (Chapman & Noller, 1977). A 50-mL culture was supplemented with 0.5–10 mCi of carrier-free  $^{32}\text{P}$  orthophosphate. Cells were harvested, washed with buffer A, and lysed by rapid freezing and thawing of the pellet, followed by grinding with an equal weight of alumina for 5 min. Ribosomal subunits were isolated according to Noller (1974), except that the sucrose gradient buffer contained 1 mM  $\text{MgCl}_2$  (buffer C).

For  $^{35}\text{S}$ -labeled ribosomes, strain MRE600 cells were grown on a low-sulfate medium as described (Sun et al., 1974). A 50-mL culture was supplemented with 10–20 mCi of carrier-free  $\text{H}_2^{35}\text{SO}_4$ . After the cells were harvested, they were lysed by the lysozyme freeze–thaw method of Ron et al. (1966) in the presence of buffer A. Ribosomal subunits were isolated as described above for  $^{32}\text{P}$ -labeled subunits.

Unlabeled 30S ribosomal proteins were isolated from *E. coli* Q13 30S subunits (gift of Dr. Ferdinand Dohme) by extraction with acetic acid (Kurland et al., 1971) and stored at –20 °C in 0.01 M Tris-HCl, pH 7.5, and 6 mM 2-mercaptoethanol.

**Modification of 30S Ribosomal Subunits with APG.** An aliquot containing 20  $\mu\text{g}$  of concentrated 30S subunits was diluted to 500  $\mu\text{L}$  with buffer E. In analytical experiments with  $^{35}\text{S}$ -labeled subunits,  $7 \times 10^4$  cpm (less than 1  $\mu\text{g}$ ) of labeled subunits were mixed with 20  $\mu\text{g}$  of unlabeled carrier subunits. In preparative experiments, 20  $\mu\text{g}$  of  $^{35}\text{S}$ -labeled subunits was used alone. A 100- $\mu\text{L}$  aliquot of 6 mg/mL APG in buffer E was added (in minus APG controls, 100  $\mu\text{L}$  of buffer E was added). Reaction mixtures were incubated at 37 °C for 45 min and then put on ice. For removal of unbound APG, subunits were precipitated twice with 0.65 volume of ethanol (–20 °C). Precipitates were resuspended in 600  $\mu\text{L}$  of buffer E for photolysis of bound APG.

**Photolysis of APG Bound to 30S Subunits.**  $^{35}\text{S}$ -Labeled subunits previously modified by APG were irradiated for up to 120 min in 1.5-mL polypropylene microcentrifuge tubes with tube caps open (Rabin & Crothers, 1979). Samples were irradiated from above in a room-temperature water bath by a 9 W, 12-in. “long-wavelength” UV tube (Edmund Scientific No. 60889), placed 2 mm above the row of sample tubes. A Pyrex petri dish could be placed between source and sample to cut off light below 300 nm.

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**Detection and Quantitation of Proteins Cross-Linked to 16S RNA by APG.** After precipitation, subunits were again precipitated with ethanol and resuspended in 50  $\mu$ L of buffer G. To measure the amount of protein bound to 16S RNA, each sample was layered on a separate 3.4-mL 5–20% sucrose gradient in buffer H. Gradients were centrifuged at 55 000 rpm for 2.5 h at 10 °C (IEC B-60 ultracentrifuge, SB-405 rotor). Gradients were fractionated, and  $^{35}$ S radioactivity was measured in each fraction.

**Characterization of Cross-Linked Proteins.** A 20- $\mu$ g sample of  $^{35}$ S-labeled 30S subunits ( $20 \times 10^6$  cpm) was reacted with APG as described above. After photolysis, non-cross-linked proteins were removed in NaDodSO<sub>4</sub>–LiCl sucrose gradients as described above, or in larger versions (35 mL, 10–30% sucrose in buffer H, 25 000 rpm for 24 h at 10 °C, SW 27 rotor). Fractions containing the center of the 16S peak were pooled. Unlabeled 30S subunits (20  $\mu$ g) were added, and the 16S RNA was precipitated with 2 volumes of ethanol after the Mg<sup>2+</sup> concentration was raised to 10 mM. After several hours at –20 °C, the RNA was pelleted (Sorvall HB-4 rotor, 8500 rpm for 4.5 h at 4 °C). The RNA pellet was redissolved in 20  $\mu$ L of water, and 100  $\mu$ L of the RNA solution was digested for 3 h at 37 °C with 10  $\mu$ L of a solution containing 0.1 mg/mL RNase T1 and 1.0 mg/mL bacterial alkaline phosphatase and 10  $\mu$ L of a 0.1 mg/mL solution of RNase A. Ribosomal proteins in the digested sample were precipitated at –20 °C with 5 volumes of acetone in the presence of 280  $\mu$ g of added unlabeled total 30S protein (*E. coli* Q13). The pellet was centrifuged out and redissolved in 50  $\mu$ L of 0.01 M Tris-HCl, pH 7.5, 6 mM 2-mercaptoethanol, and 8 M urea.

Proteins were separated by using the two-dimensional polyacrylamide–urea electrophoresis system of Kaltschmidt & Wittmann (1970). After electrophoresis, the gel was fixed in 5% trichloroacetic acid for 1 h and stained in 0.25% Coomassie Blue in 9.2% acetic acid and 45% methanol. After the gel was destained, it was impregnated with PPO according to Bonner & Laskey (1974) or was soaked in 90 mL of Enhance (New England Nuclear) for 2.5 h at 20 °C and then in water at 20 °C for 1 h. The gel was then dried overnight and fluorographed on Kodak XR-5 film for 4–45 days at –80 °C.

**Detection of Cross-Linked Oligonucleotide–Protein Complexes.** A 20- $\mu$ g sample of  $^{32}$ P-labeled 30S subunits ( $20 \times 10^6$  cpm) was cross-linked with APG as described above. After resuspension in 50  $\mu$ L of buffer G, RNA in the sample was precipitated with 2.5 volumes of ethanol in the presence of 20  $\mu$ g of added carrier 30S subunits (MRE600). The RNA pellet was digested for 1 h at 37 °C with 25  $\mu$ L of a solution containing 1.2  $\mu$ g of RNase T1, 12  $\mu$ g of bacterial alkaline phosphatase, and 1.2  $\mu$ g of RNase A. As above, ribosomal proteins and cross-linked nucleotides were concentrated by acetone precipitation in the presence of carrier MRE600 total 30S proteins, and the mixture was separated by two-dimensional electrophoresis. The destained gel was dried overnight and autoradiographed on Kodak XR-5 film for 7 days at –80 °C.

**Quantitation of Modified Guanosine in APG-Modified 30S Subunits.** Subunits labeled with  $^{32}$ P were modified with APG as described for  $^{35}$ S-labeled subunits. Each sample contained 20  $\mu$ g of subunits ( $60 \times 10^6$  cpm). After the samples were reacted with APG (without UV photolysis), subunits were ethanol precipitated, resuspended in buffer H, and deproteinized in NaDodSO<sub>4</sub>–LiCl sucrose gradients as described for  $^{35}$ S-labeled subunits. The 16S peak fractions were pooled, and then RNA was recovered by ethanol precipitation and digested completely with RNase T2 (Barrell, 1971). The

Table I: Thin-Layer Chromatography Results<sup>a</sup>

compound	<i>R<sub>f</sub></i>
guanosine	0.50
kethoxal–guanosine product	0.84
APG–guanosine adduct	0.88

<sup>a</sup> Reactions and chromatography are as described under Experimental Section.

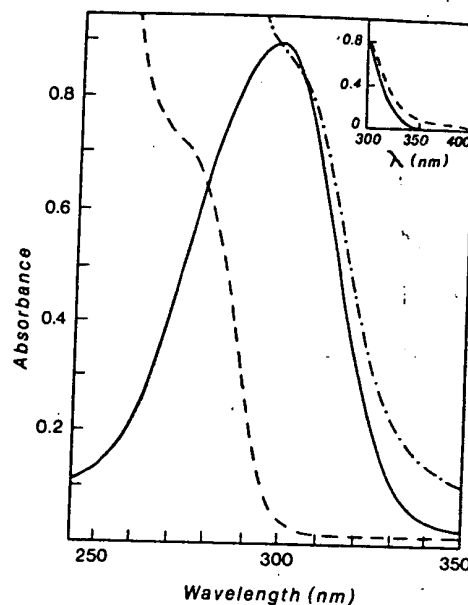


FIGURE 1: Ultraviolet spectra of guanosine (---), APG (—), and equimolar guanosine plus APG (.....). All samples were incubated at room temperature for 24 h in buffer E at final equimolar concentrations of  $1.67 \times 10^{-4}$  M guanosine and APG. Samples were diluted to  $3.3 \times 10^{-5}$  M with buffer E just before recording spectra. Inset: Visible spectra of a 24-h reaction mixture of equimolar APG and guanosine (---) vs. an equimolar mixture of APG and guanosine just after mixing (—). Both samples were  $3.3 \times 10^{-5}$  M when spectra were recorded.

digestion products were separated by electrophoresis at pH 1.7 (7% formic acid, Whatman 3MM paper) for 45 min at 2000 V. After autoradiography, the unmodified mononucleotide and APG-modified spots were cut out and counted.

## Results

The reagent (4-azidophenyl)glyoxal was obtained as the crystalline monohydrate in a three-step synthesis. APG was stable when stored as the solid at –20 °C for at least 18 months, as judged by thin-layer chromatography.

Reaction of APG with ribonucleosides and ribonucleoside monophosphates was examined by thin-layer chromatography, paper electrophoresis, and UV spectroscopy. Initially, reaction between guanosine and APG was compared to the reaction between guanosine and kethoxal, a well-characterized reagent for guanosine modification in nucleic acids. Reaction of either APG or kethoxal with guanosine showed partial conversion of guanosine to a less polar product (Table I). The APG–guanosine adduct showed up as an intense green-yellow fluorescent spot when viewed under UV light.

Ultraviolet and visible spectra of APG–guanosine reaction mixtures were compared with spectra of APG and guanosine (Figure 1). After 24 h at room temperature, the reaction mixture showed a pronounced shoulder or tail (approximately 10% of the absorbance at 300 nm) compared with a spectrum of APG at the same molar concentration. This shoulder extended the reaction mixture spectrum out to about 400 nm

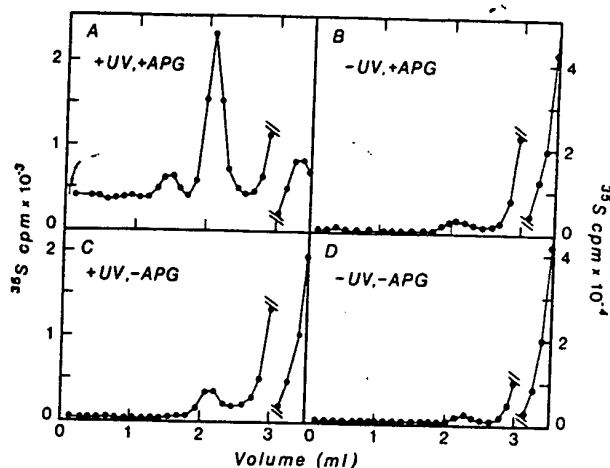


FIGURE 2: NaDodSO<sub>4</sub>-LiCl sucrose gradient analysis of RNA-protein cross-linking in 30S subunits by APG. Details of chemical modification and sedimentation are described under Experimental Section. Each sample contained  $7 \times 10^4$  cpm of <sup>35</sup>S radioactivity. Sedimentation was from right to left. Samples were derived from subunits that were (A) APG modified and UV photolyzed; (B) APG modified only; (C) UV photolyzed only; (D) unmodified. Photolysis in (A) and (C) was for 120 min.

whereas the APG spectrum showed zero absorbance in the region 350–400 nm. The inset to Figure 1 demonstrates that the shoulder is due to a reaction product, since a spectrum of guanosine plus APG taken immediately after mixing lacks this feature.

The specificity of APG for guanine was demonstrated in reaction with purified <sup>32</sup>P-labeled nucleoside monophosphates. Reaction products were separated by paper electrophoresis, pH 3.5. A modified spot appeared in the GMP-APG reaction mixture, migrating as an elongated spot between the positions of GMP and AMP. After reaction for 1 h at 37 °C with an excess of APG, 10.4% of the total radioactivity was converted to the modified spot. No modified spots were detected by autoradiography in similar reactions with UMP, AMP, and CMP.

Reaction of APG with guanylic acid residues in 30S subunits was measured. After dark reaction with APG, 16S RNA labeled with <sup>32</sup>P was extracted from 30S subunits and digested to mononucleotides with RNase T2. The mixture of mononucleotides was separated by paper electrophoresis at pH 1.7 (Bellemare et al., 1972). In this system, APG-modified guanylic acid has a mobility of 1.5 times that of guanylic acid. Compared to a control in which reagent was omitted, 0.26% of the total radioactivity appeared as modified guanylic acid, corresponding to an average value of 4 mol of guanosine modified per mol of 30S subunits.

When <sup>35</sup>S-labeled 30S subunits were modified with APG and then irradiated with ultraviolet light, proteins were cross-linked to 16S RNA. Cross-linking was measured as the amount of <sup>35</sup>S-labeled protein which comigrated with 16S RNA in NaDodSO<sub>4</sub>-LiCl sucrose gradients (Figure 2). Such gradients rapidly and cleanly separate noncovalently bound ribosomal protein from 16S RNA. For example, a background of only 0.34% of total input radioactivity comigrated with the 16S peak when unmodified, unirradiated subunits were sedimented in this fashion (Figure 2D). In contrast, when subunits previously modified with APG were irradiated with UV light, 8.7% of the <sup>35</sup>S radioactivity comigrated with the 16S peak (Figure 2A). A small peak of radioactivity sedimenting faster than 16S and a higher background at the bottom of the gradient were usually observed with cross-linked subunits (e.g., Figure 2A). These effects are probably the result of cross-

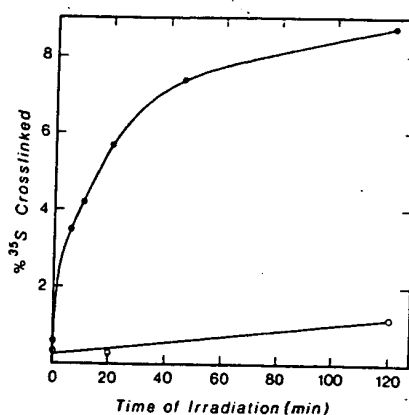


FIGURE 3: Time course of UV-dependent RNA-protein cross-linking. Percent cross-linking was measured in the standard NaDodSO<sub>4</sub>-LiCl sucrose gradient assay. See Experimental Section for details. Each sample contained  $7 \times 10^4$  cpm of <sup>35</sup>S radioactivity. (●) APG-modified subunits; (○) unmodified subunits.

linking the 30S–30S dimers or larger aggregates present at the 10 mM Mg<sup>2+</sup> concentration in the reaction mixture. The appearance of higher aggregates at the expense of the 16S peak strongly depended on the reagent concentration; in order to optimize the yield in the 16S peak with respect to the larger aggregates, it was necessary to set the APG concentration for the modification reaction maximally at 1 mg/mL (data not shown).

Cross-linking was dependent on both treatment with APG and irradiation. Reaction with APG without subsequent irradiation caused only 0.60% of the <sup>35</sup>S radioactivity to comigrate with the 16S peak (Figure 2B) while irradiation in the absence of APG modification caused 1.14% comigration with the 16S peak (Figure 2C). This small amount of UV-dependent cross-linking could be reduced to background by irradiation for 20 min instead of the 120 min of Figure 2 (see Figure 3 and the following).

The kinetics of cross-linking were followed by using the sucrose gradient assay. Identical samples were modified with APG and then photolyzed for different lengths of time. Each sample was sedimented on a standard NaDodSO<sub>4</sub>-LiCl sucrose gradient, and <sup>35</sup>S radioactivity in the 16S peak was measured. The time course of the cross-linking reaction is biphasic (Figure 3). The first, rapid phase is finished by approximately 30 min, after which cross-linking occurs only at a very slow rate, similar to that seen when unmodified subunits are irradiated (Figure 3, lower curve). Insertion of a Pyrex filter between UV source and sample to absorb light less than 300 nm in wavelength caused no additional reduction of background after 20 min of irradiation (data not shown). Because of the small amount of UV-dependent cross-linking observed after 120 min of photolysis (Figure 2C and Figure 3, lower curve), in subsequent preparative experiments, APG-modified subunits were photolyzed for only 20 min.

The <sup>35</sup>S-labeled proteins which comigrate with 16S RNA after APG modification and photolysis were isolated by using a preparative version of the NaDodSO<sub>4</sub>-LiCl sucrose gradient. Fractions containing <sup>35</sup>S radioactivity in the 16S peak were pooled and concentrated; the material was then subjected to total RNase digestion and analysis by two-dimensional gel electrophoresis in the presence of stainable amounts of total 30S protein. Results of a typical experiment are shown in the fluorogram of Figure 4. Five radioactive spots correspond closely in position to the stained spots for S2, S3, S4, S5, and S12. Each of these radioactive spots overlaps with its corresponding stained protein and streaks toward the first-dimension

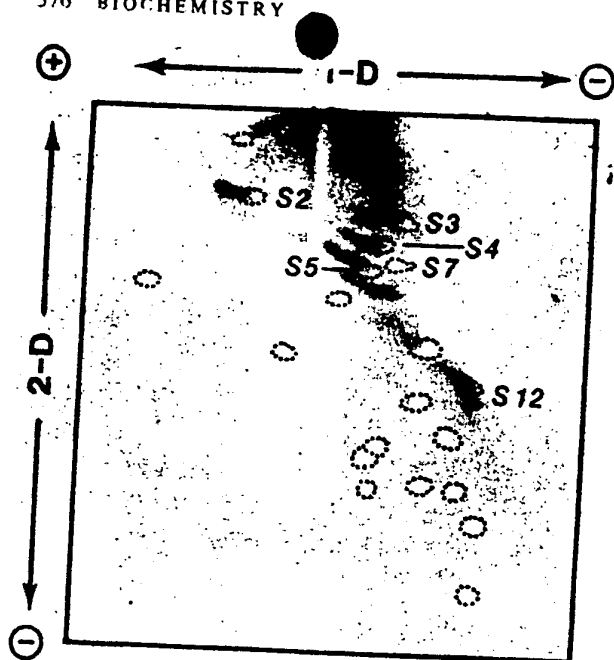


FIGURE 4: Fluorogram of a two-dimensional polyacrylamide electrophoresis gel of the  $^{35}\text{S}$ -labeled 30S proteins which are cross-linked to 16S RNA by APG. Details are described under Experimental Section. The diagram shows the positions of unlabeled stained 30S proteins from strain Q13 (dashed spots). Cross-linked proteins are identified by number.

mode. The overall pattern of radioactive spots is clearly homologous to the corresponding pattern of stained spots. A sixth spot, most likely corresponding to protein S7, runs anomalously in a position below that of the stained spots for S5 and S7. This can be attributed to the well-documented difference in mobility of protein S7 derived from strain MRE600 ( $^{35}\text{S}$ -labeled proteins) and strain Q13 (stained proteins). The right-hand edge of the sixth spot corresponds closely to the position of protein S7 from strain MRE600 (Kaltschmidt et al., 1970; see also Figure 5).

In Figure 4, some  $^{35}\text{S}$ -labeled cross-linked material migrates too slowly to be single 30S proteins. This material is probably not a large complex of both RNA and protein (see the same region of the gel in Figure 5). It could represent 30S proteins cross-linked to each other via APG-modified arginyl residues.

Four separate analyses of the cross-linked proteins were conducted. In one of the analyses, RNA was digested with RNase T2; in the other three, including the experiment of Figure 4, RNA was digested with a mixture of RNase T1, pancreatic RNase, and alkaline phosphatase. In each case, a similar pattern of fluorographed spots was observed (data not shown).

The experiment of Figure 4 was repeated, using  $^{32}\text{P}$ -labeled subunits, to determine whether proteins from APG-cross-linked ribosomes contain covalently linked nucleotides which cannot be removed by RNase digestion. In the autoradiogram shown in Figure 5, a pattern of  $^{32}\text{P}$ -labeled spots similar to that of the  $^{35}\text{S}$ -labeled pattern of Figure 4 is found. In particular,  $^{32}\text{P}$  spots homologous to the positions of stained proteins S2, S3, S4, S5, and S7 are evident. As in Figure 4, the labeled spots are elongated in the direction of the anode. In this case, the displacement of the  $^{32}\text{P}$  spots from the stained pattern is exaggerated, possibly because the intensity of the label reflects the length of the attached RNA fragment as well as the relative amount of cross-linked complex. A few faster migrating  $^{32}\text{P}$ -labeled spots can be seen which are diffuse and thus less easily related to the staining-pattern.

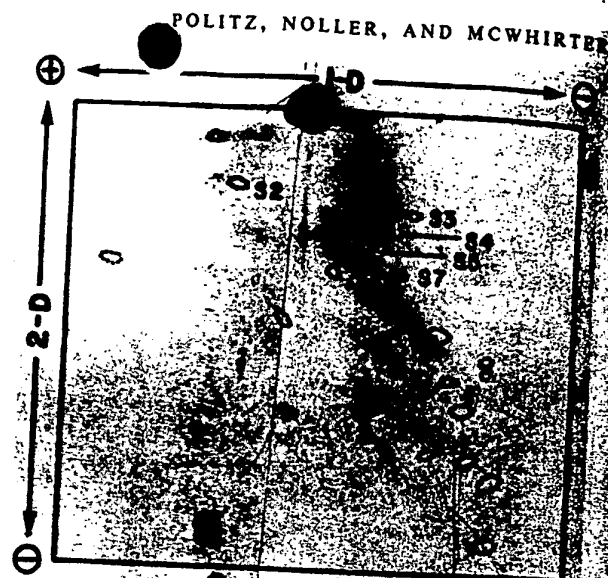


FIGURE 5: Autoradiogram of two-dimensional electrophoresis of  $^{32}\text{P}$ -labeled nucleotides bound to 30S ribosomal proteins after APG cross-linking of  $^{32}\text{P}$ -labeled subunits. Details are described under Experimental Section. The diagram shows the position of unlabeled stained 30S proteins from strain MRE600 (dashed spots). Cross-linked proteins are identified by number. The figure is a composite of two (left and right) autoradiograms of the same electrophoresis gel.

#### Discussion

The specificity of reaction of APG with guanosine and guanosine monophosphate is similar to that of other dicarbonyl compounds (e.g., glyoxal, phenylglyoxal, and kethoxal). The guanosine-APG adduct is fluorescent, like the guanosine-phenylglyoxal adduct reported by Shapiro & Hachmann (1966).

We anticipated reaction of APG with 30S ribosomal subunits to occur between guanylic acid residues of 16S RNA and the dicarbonyl group of APG. That this takes place is demonstrated by the detection of a modified guanosine monophosphate in APG-modified,  $^{32}\text{P}$ -labeled 30S subunits. Quantitatively, approximately four guanylic acid residues are modified per 30S subunit. It was important to estimate guanosine modification by APG because phenylglyoxal is a well-known chemical modification reagent for arginine residues in proteins (Takahashi, 1968). Although we have not attempted to measure APG modification of arginine in 30S proteins, our conditions have been chosen to minimize arginine modification. The rate constant for arginine modification by phenylglyoxal increases 40-fold with increasing pH in the range 7.5–11.5 (Takahashi, 1968, 1977; Cheung & Fonda, 1979) and decreases 12-fold in the presence of 50 mM borate ion (Cheung & Fonda, 1979). Conversely, the reaction of dicarbonyl compounds with guanosine is promoted by borate and neutral pH (Litt, 1969). Our conditions with respect to these variables are pH 7.0 and 0.02 M sodium borate, respectively.

Cross-linking of 30S proteins to 16S RNA occurs when APG-modified 30S subunits are irradiated with UV light. That this cross-linking involves photolysis of bound APG is suggested by the conditions required; i.e., both irradiation and prior APG modification are required for cross-linking. Irradiation alone produces no cross-linking, indicating that nucleic acids and proteins are probably not photolyzed by the irradiation conditions used. APG treatment without irradiation also produces no cross-linking, suggesting that no unexpected chemistry is involved.

The cross-linking yield (ca. 5%) represents roughly one protein molecule cross-linked per 16S RNA molecule or 30S



subunit, assuming an approximately homogeneous distribution of  $^{35}\text{S}$  radioactivity in the 21 30S proteins. It is probably not desirable to promote more extensive cross-linking since this could result in disruption of the native ribosome structure.

After isolation of  $^{35}\text{S}$ -labeled cross-linked proteins and digestion of the cross-linked RNA, most of the  $^{35}\text{S}$  radioactivity migrates in discrete protein spots on two-dimensional electrophoresis gels, corresponding to a specific subset of the 30S ribosomal proteins. The  $^{35}\text{S}$ -labeled spots are elongated in the direction of the anode, suggesting that the labeled proteins are covalently linked to residual tails of RNase-resistant oligonucleotides. This interpretation is confirmed by the experiment of Figure 5, which demonstrates that  $^{32}\text{P}$ -labeled nucleotides are found in protein spots from APG-cross-linked 30S subunits on two-dimensional gels. Furthermore, the  $^{32}\text{P}$ -labeled spots are shifted in the expected direction relative to the corresponding stained spots, i.e., toward the anode. Since the second-dimension electrophoresis (pH 4.5) is run toward the cathode, these  $^{32}\text{P}$ -labeled spots cannot be free oligonucleotides.

The altered electrophoretic mobility of ribosomal proteins bearing negatively charged phosphates is well documented, in the field of both protein phosphorylation (Gressner & Wool, 1974) and ribosomal RNA-protein cross-linking (Möller & Brimacombe, 1975). The altered mobilities observed here in Figures 4 and 5 constitute strong evidence that RNA-protein cross-linking has occurred.

The  $^{35}\text{S}$ -labeling results (Figure 4) suggest that proteins S2, S3, S4, S5, S7, and S12 are cross-linked to 16S RNA by APG. Cross-linking of S2, S3, S4, S5, and S7 is confirmed by the  $^{32}\text{P}$ -labeling results of Figure 5. Of these proteins, S4 and S7 have been previously cross-linked to 16S RNA by other RNA-protein cross-linking techniques. It is not surprising that S4 is found as a protein cross-linked to 16S RNA, since it interacts strongly with 16S RNA [reviewed in Zimmermann (1980)] and may by itself have a great deal to do with stabilizing the shape of the 30S subunit (Garrett, 1979). S7 also binds to 16S RNA in the absence of other proteins (Nomura & Held, 1974), protects a large region of 16S RNA from RNase digestion (Muto et al., 1974), and has been cross-linked to U<sub>1239</sub> of 16S RNA by UV irradiation (Möller et al., 1978). The other cross-linked proteins described here, S2, S3, S5, and S12, are poorly understood in terms of their interaction with RNA. Thus, APG cross-linking of these proteins to 16S RNA offers the opportunity to learn a great deal about ribosome structure. We are presently determining which sequences in 16S RNA are cross-linked to the various 30S proteins by APG. It may be possible to use this information in conjunction with ribosomal protein topography data to locate regions of the RNA chain in a three-dimensional model of the 30S subunit.

Besides S4 and S7, the other cross-linked proteins also deserve further mention because of their implication as components of ribosomal functional sites. Proteins S2 and S3 have been shown to be important for tRNA binding to the ribosome (Rummel & Noller, 1973; Thomas et al., 1975; Van Duin et al., 1972; Randall-Hazelbauer & Kurland, 1972). Protein S12 is altered in streptomycin-resistant and -dependent *E. coli* strains (Ozaki et al., 1969; Birge & Kurland, 1970); protein S5 is altered in spectinomycin-resistant mutants and in resistant from streptomycin dependence (Hasenbank et al., 1973; Bollen et al., 1969). Lake (1980) has suggested that S5 and S12 may be located at the tRNA recognition site of the 30S subunit. Of course, information about all the cross-linking sites will be important, but the identification of parts of 16S RNA which are near known-ribosomal functional sites will be particularly interesting.

The potential of APG for structural analysis of nucleoproteins must, of course, be proven by application. These initial studies indicate that APG modification is promising as a general method for cross-linking nucleic acids to proteins in biological systems. It is applicable wherever guanosines in either DNA or RNA are in single-stranded conformations which are accessible to the dicarbonyl group. APG reacts under mild conditions of pH and temperature. The lack of specificity of the nitrene generated on photolysis is a valuable property, since one seldom knows the type of chemical groups which are present in the target environment. As a consequence, APG may also be useful as an RNA-RNA cross-linking reagent for studying such problems as the tertiary structure of the large ribosomal RNA molecules.

#### Acknowledgments

We thank Dr. David Sigman for suggesting the use of a photoinducible cross-linking reagent, Dr. Ferdinand Dohme for a gift of unlabeled ribosomal subunits and advice on two-dimensional gel electrophoresis, and Dr. Alexei Kopylov, Dr. Jürgen Brosius, Dr. Roger Garrett, Joan Politz, and K. Katze for helpful discussions.

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## Lipids of Synaptic Vesicles: Relevance to the Mechanism of Membrane Fusion<sup>†</sup>

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**ABSTRACT:** Synaptic vesicles from the electric organ of the marine ray *Narcine brasiliensis*, purified to at least 90% homogeneity, were analyzed for the lipid and fatty acid content of their membranes. The major lipids (mol %) were phosphatidylcholine (32.3%), phosphatidylethanolamine (20.5%), phosphatidylserine (6.1%), sphingomyelin (3.0%), and cholesterol (33.3%), a composition which did not differ greatly from that of the parent electric organ. While the number of double bonds per fatty acid molecule was similar for both synaptic

vesicle and whole electric organ phospholipids, the vesicles were highly enriched in docosahexenoic acid (22:6). Reaction with the amine labeling reagents isethionylacetimidate and trinitrobenzenesulfonic acid indicated that 40% of the phosphatidylserine and 60% of the phosphatidylethanolamine are present on the external (cytoplasmic) surface of the synaptic vesicle. These data on a natural fusing membrane have relevance to models of membrane fusion, which have been based largely on studies of in vitro fusion using synthetic membranes

In many types of secretory cells the secretory product is contained in membrane vesicles or granules and the release of the secretory product involves the fusion of the vesicle with the cell plasma membrane. The vesicle membrane constituents

are at least transiently incorporated into the plasma membrane, and the contents are released into the extracellular space. In general this process of exocytosis is triggered by entry of calcium into the cell (Douglas, 1968; Llinas & Heuser, 1977; Holtzman, 1977). Exocytosis in vivo may involve proteins of the vesicle membrane and plasma membrane or the carbohydrate moieties of their glycoproteins and glycolipids. In vitro, however, model membrane studies [review, Papahadjopoulos et al. (1979)] have shown that membranes composed solely of purified lipids are capable of undergoing fusion. It has been

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9. *2,2'-Dichlor-benzoin*: Analog der 4,4'-Verbindung. 1.380 g Einschlußverb., Lösungstemp. 55°, eingeschl. 0.200 g;  $[\alpha]_D^{25}$ :  $+0.06 \cdot 5/1 \cdot 0.200 = +1.50^\circ$  (in Essigester).
10. *Benzoinmethyläther*: Analog zum 4,4'-Dichlor-benzoin, nur wurde hier nach Abkühlen auf 20° noch einige Zeit bei 0° gerührt. 1.528 g Einschlußverb., Lösungstemp. 45°, eingeschl. 0.229 g;  $[\alpha]_D^{25}$ :  $+0.036 \cdot 5/1 \cdot 0.229 = +0.79^\circ$  (in Äthanol), entspr. 0.84 % Aktivierung.
11. *Zimtsäuredibromid*: 1.5 g  $\beta$ -Dextrin wurden in 100 ccm Wasser gelöst und mit einer Lösung von 2.05 g Dibromid (5fach molarer Überschuß) in 50 ccm Äther 7 1/2 Stdn. bei 0° gelegentlich geschüttelt. 1.359 g Einschlußverb., Lösungstemp. 70°. Zur Isolierung des Gastes wurde Trichloräthylen in Mischung 1:1 mit Essigester verwendet; eingeschl. 0.062 g;  $[\alpha]_D^{25}$ :  $+0.158 \cdot 3/1 \cdot 0.062 = +7.71^\circ$  (in Äthanol), entspr. 11.33 % Aktivierung.
12. *Menthol*: Die  $\beta$ -Dextrinlösung wurde mit einer Lösung von 0.62 g Menthol (3fach molarer Überschuß) in 10 ccm Äther unter Eiskühlung mehrfach geschüttelt, sodann 24 Stdn. im Eisschrank stehengelassen. 1.166 g Einschlußverb., Lösungstemp. 55°, eingeschl. 0.084 g;  $[\alpha]_D^{25}$ :  $-0.066 \cdot 3.093/1 \cdot 0.084 = -2.44^\circ$  (in Äthanol), entspr. 4.88 % Aktivierung.
13. *Dibrombernsteinsäure*: In einer Lösung von 1.5 g  $\beta$ -Dextrin in 60 ccm Wasser wurden 1.8 g (5fach molarer Überschuß) der Säure gelöst und noch 10 ccm Äther zugegeben. Das Ganze wurde 5 1/2 Stdn. bei 0° gelegentlich geschüttelt. 1.009 g Einschlußverb., Lösungstemp. 40°. Die Einschlußverb. wurde in 50 ccm Wasser gelöst. Statt reinem Trichloräthylen wurde dieses in Mischung 1:1 mit Essigester verwendet; eingeschl. 0.050 g;  $[\alpha]_D^{25}$ :  $+0.200 \cdot 3/1 \cdot 0.050 = +12.10^\circ$  (in Äthanol), entspr. 8.18 % Aktivierung.
14. *Atrolactinsäure*: In einer Lösung von 1.5 g  $\beta$ -Dextrin in 100 ccm Wasser wurden 1.1 g (5fach molarer Überschuß) Atrolactinsäure gelöst und noch 5 ccm Äther zugegeben. Die Lösung wurde 3 1/2 Stdn. bei 0° gelegentlich geschüttelt. 1.182 g Einschlußverb., Lösungstemp. 45°. Aufbereitung der Einschlußverb. wie bei Dibrombernsteinsäure; eingeschl. 0.073 g;  $[\alpha]_D^{25}$ :  $-0.07 \cdot 3/1 \cdot 0.073 = -2.90^\circ$  (in Wasser), entspr. 5.57 % Aktivierung.

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### Eine Synthese der 6-Desoxy-6-amino-glucose

Aus dem Chemischen Institut der Universität Heidelberg  
(Eingegangen am 20. Oktober 1958)

Glucoside lassen sich ohne Maskierung der sek. Hydroxyle mit 1 Mol. Tosyl- (bzw. Mesyl-)chlorid direkt in die entsprechenden 6-Tosyl- (bzw. Mesyl-) Derivate überführen, die mit Ammoniak zu den 6-Amino-, mit Natriumazid zu den 6-Azido-Derivaten reagieren. Letztere können zu den 6-Amino-Derivaten reduziert werden, in denen sich die Glucosidbindung jedoch nur schwer spalten läßt. Durch saure Hydrolyse der Monoaceton-6-acetyl-amino-glucose erhält man die 6-Amino-glucose in Form ihres Acetylderivates.

Unter den Aminoglucosen ist die *2-Desoxy-2-amino-glucose* die in der Natur am weitesten verbreitete; eine allgemein anwendbare Synthese dieses und anderer 2-Aminozucker ist von R. KUHN<sup>1)</sup> beschrieben worden. Die *3-Desoxy-3-amino-*



glucose, die bisher nicht in der Natur aufgefunden wurde, läßt sich nach FREUDENBERG durch Aminolyse der 1.2-Isopropyliden-3-tosyl-glucose bereiten<sup>2,3)</sup>. 6-Desoxy-6-amino-glucose wurde kürzlich als Bestandteil des Antibiotikums *Kanamycin*, eines Trisaccharides, aufgefunden<sup>4)</sup>.

Erste Versuche zur Synthese dieser Verbindung stammen von E. FISCHER<sup>5)</sup>. Derivate der 6-Amino-glucose haben OHLE und Mitarbb.<sup>6-8)</sup> durch Umsetzung der 1.2-Isopropyliden-6-tosyl-glucose mit methanol. Ammoniak gewonnen. Da der Ersatz einer Tosylgruppe durch eine Aminogruppe in Zuckern mit freier  $\alpha$ -ständiger Hydroxylgruppe über Anhydroderivate führen kann<sup>7,9)</sup> (z. B. 5.6-Anhydro-), die anschließend unter Konfigurationsumkehr geöffnet werden können<sup>3,10,11)</sup>, muß die Konstitution der 6-Amino-glucose von Ohle jedoch als noch nicht völlig gesichert betrachtet werden. Nach B. HELFERICH<sup>12)</sup> läßt sich auch ein Mesylester, nämlich 1.2-Isopropyliden-3.5-benzal-6-mesyl-D-glucose zur entsprechenden Aminoverbindung umsetzen.

Wir suchten nach einfachen Methoden, um Derivate von 6-Desoxy-6-amino-glucopyranosiden-(1.5) darzustellen, besonders im Hinblick auf die Einführung der Aminogruppe in die 6-Stellung der Cyclodextrine<sup>\*)</sup>.

#### 1. 6-TOSYL- (BZW. MESYL-)- $\alpha$ -METHYL- (BZW. BENZYL-)-GLUCOSID<sup>13)</sup>

Das primäre Hydroxyl eines Zuckers ist bekanntlich gegenüber Acylierungs- und Alkylierungsreagenzien bei weitem am reaktionsfähigsten<sup>14-18)</sup>. Es erschien uns daher aussichtsreich, die 6-Tosylester geeigneter Glucosederivate direkt darzustellen und anschließend die Tosylgruppe zu substituieren<sup>19)</sup>.

1938 hat J. COMPTON<sup>20)</sup>  $\alpha$ -Methylglucosid mit 1 Mol. Tosylchlorid umgesetzt, die Konstitution des Reaktionsproduktes ließ sich jedoch nur indirekt beweisen. 1946 konnte

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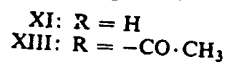
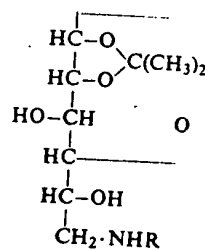
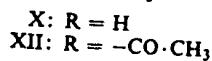
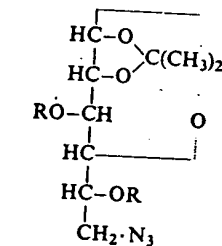
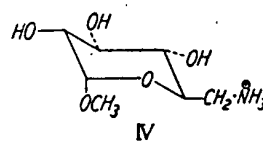
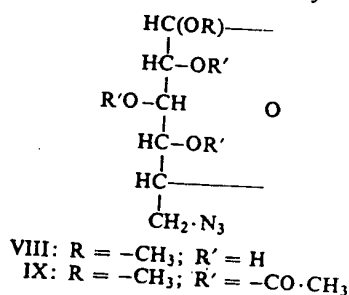
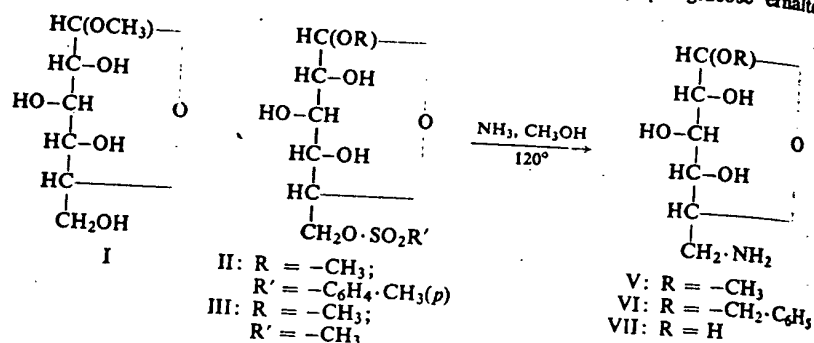
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HARDEGGER<sup>21)</sup> in Analogie zu HELFERICH<sup>22)</sup> zeigen, daß bei der direkten Tosylierung und nachfolgenden Acetylierung von Glucose 1.2.3.4-Tetraacetyl-6-tosyl- $\beta$ -D-glucose erhalten werden kann.



$\alpha$ -Methylglucosid (I) reagierte mit 1 Mol. Tosylchlorid direkt zum schön kristallisierten 6-Tosylester II, den HAWORTH und HELFERICH<sup>23)</sup> auf anderem Wege gewonnen haben; die Substanz verbraucht 2 Moll. Perjodat, womit die Acylierung in 6-Stellung bewiesen ist. Die Mesylierung von I führt in Übereinstimmung mit HELFERICH<sup>22)</sup> nur zu einem Sirup (III), der sich in das kristallisierte Acetylderivat überführen läßt.  $\beta$ -Benzylglucosid<sup>24)</sup> ergab mit Tosylchlorid in gleicher Weise das 6-Tosylderivat.

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2. AMINOLYSE DER 6-TOSYLESTER<sup>13)</sup>

II reagierte mit methanol. Ammoniak bei 120° in 16 Stdn. zum 6-Desoxy-6-amino-methyl- $\alpha$ -D-glucosid (V), welches nach Aufbereitung über Ionenaustauscher in Form des kristallisierten Hydrochlorides isoliert werden konnte. V widerstand jedoch hartnäckig der sauren Hydrolyse zum freien Zucker. Das Studium des Reaktionsverlaufes mittels Papierchromatographie und Methoxylbestimmung zeigte, daß selbst nach 3stdg. Erwärmen mit 1.5*n* HCl im Rohr auf 100°, Bedingungen, die schon zu starker „Bräunung“ führen, noch 20 % Glucosid vorhanden sind. Die Methode kann also wie bei FISCHER und ZACH<sup>5)</sup> nicht zu einem reinen Material führen. Zweifellos ist für die große Stabilität von V die elektrostatische Abschirmung des Glykosid-Sauerstoffs durch die positive Ladung des Ammoniumions IV verantwortlich<sup>25)</sup>, die hier größer ist als bei jeder anderen Aminoglucose. Im  $\beta$ -Benzylglucosid ließ sich die 6-Tosylgruppe ebenfalls leicht durch die Aminogruppe ersetzen (VI). Durch Hydrieren des salzsauren Salzes von VI erhielten wir die 6-Desoxy-6-amino-glucose (VII) in Form des amorphen Hydrochlorides. Die Substanz war im Chromatogramm einheitlich und reduzierte Fehlingsche Lösung, lieferte jedoch kein Phenylhydrazon und keine Anisalverbindung.

3. 6-AZIDO-ZUCKER<sup>26)</sup>

Alkylhalogenide und Sulfonsäureester lassen sich durch Reaktion mit Azid-Anion in die Azidoverbindungen überführen. In der Zuckerreihe ist die Reaktion unseres Wissens bisher nur mit den reaktionsfähigen 1-Halogen-Zuckern durchgeführt worden<sup>27)</sup>, sie gelang damals jedoch nicht mit einem Derivat der 6-Desoxy-6-brom-glucose. Es gelang uns, II durch Kochen mit Natriumazid in wäBr. Aceton in VIII überzuführen, welches durch das Triacetylderivat IX charakterisiert wurde. Direkte Hydrierung (s. u.) von VIII lieferte das Aminoglucosid V, welches mit dem nach dem früheren Verfahren gewonnenen identisch war. 1.2-Isopropyliden-6-tosyl-(bzw. mesyl-)glucofuranose<sup>28,29)</sup> reagierten ebenfalls — am besten in einer Mischung von Aceton/Wasser (5:1) — in 67-proz. Ausbeute zur 1.2-Isopropyliden-6-desoxy-6-azido-glucofuranose (X). Dicyclohexylammoniumazid<sup>30)</sup> erwies sich für diesen Fall als dem Natriumazid unterlegen.

4. HYDRIERUNG DER 6-AZIDO- ZU DEN 6-AMINO-ZUCKERN<sup>31)</sup>

Organische Azide lassen sich katalytisch zu Aminen und elementarem Stickstoff hydrieren<sup>32-35)</sup>; auf diese Weise ergaben VIII und IX in glatter Reaktion das Amin V.

<sup>25)</sup> R. C. MOGGRIDGE und A. NEUBERGER, J. chem. Soc. [London] 1938, 745; G. N. RICHARDS, Chem. and Ind. 1955, 228; J. T. EDWARDS, ebenda 1955, 1102; G. HUBER, Helv. chim. Acta 38, 1224 [1955].

<sup>26)</sup> Diplomarb. H. SPRINGMANN, Univ. Heidelberg 1956.

<sup>27)</sup> A. BERTHO, Ber. dtsh. chem. Ges. 63, 836 [1930]; A. BERTHO und A. RÉVÉSZ, Liebigs Ann. Chem. 581, 161 [1953].

<sup>28)</sup> H. OHLE und E. DICKHÄUSER, Ber. dtsh. chem. Ges. 58, 2602 [1925].

<sup>29)</sup> K. FREUDENBERG und K. v. OERTZEN, Liebigs Ann. Chem. 574, 37 [1951].

<sup>30)</sup> F. WEYGAND und M. REIHER, Chem. Ber. 88, 31 [1955].

<sup>31)</sup> Dissertat. H. SPRINGMANN, Univ. Heidelberg 1957.

<sup>32)</sup> H. WIENHAUS und H. ZIEHL, Ber. dtsh. chem. Ges. 65, 1461 [1932].

<sup>33)</sup> A. BERTHO und J. MAIER, Liebigs Ann. Chem. 495, 113 [1932].

<sup>34)</sup> K. FREUDENBERG, H. EICHEL und F. LEUTERT, Ber. dtsh. chem. Ges. 65, 1183 [1932].

<sup>35)</sup> A. BERTHO und J. MAIER, Liebigs Ann. Chem. 498, 50 [1932].

Aus X erhielten wir das kristallisierte Hydrochlorid der 1.2-Isopropyliden-6-desoxy-6-amino-glucose (XI). Bei der sauren Abspaltung des Isopropylidenrestes wurde der freie Zucker VII jedoch ebenfalls nur als leicht zersetzliche, amorphe Substanz erhalten. Um zu einem beständigen Derivat von VII zu gelangen, wurde X in die Diacetylverbindung XII übergeführt und diese mit Raney-Nickel hydriert. Dabei wandert der Acylrest aus der 5-Stellung der Glucose sofort an die 6-Aminogruppe (Auftreten der Amid-I- und Amid-II-Bande im IR bei 1650 und 1540/cm), die 3-Acetylgruppe wird unter den schwach alkalischen Reaktionsbedingungen in Methanol abgespalten, und wir erhielten sofort XIII. Wegen der abgeschwächten Basizität des Stickstoffs konnte XIII nunmehr leicht in 5 Tagen mit Schwefelsäure bei  $pH$  1.5 und 25° zur *N*-Acetyl-6-amino-glucose<sup>3)</sup> hydrolysiert werden.

Die Arbeit wurde von der DEUTSCHEN FORSCHUNGSGEMEINSCHAFT und dem FONDS DER CHEMIE unterstützt.

#### BESCHREIBUNG DER VERSUCHE

1. *6-Tosyl-methyl- $\alpha$ -D-glucosid (II)*: 20 g  *$\alpha$ -Methylglucosid*, in 180 ccm absol. Pyridin gelöst, wurden bei 0° innerhalb von 20 Min. unter Rühren mit der Lösung von 21 g *p*-Toluolsulfonsäurechlorid in 50 ccm Pyridin versetzt. Dann wurde 2 Tage bei 20° gehalten und anschließend das Pyridin bei 40° i. Vak. abgedampft. Der Rückstand wurde in Chloroform aufgenommen, mit Kaliumhydrogensulfat- und Natriumhydrogencarbonatlösung gewaschen und die Chloroformlösung getrocknet. Nach Abdampfen des Chloroforms hinterblieben 30 g eines Sirups, die in 260 ccm Benzol unter Rückfluß gelöst wurden. Beim Abkühlen erstarrte die Lösung zu einem gelartigen Brei, der abgesaugt und im Exsikkator über Paraffintrockenen Pulvers 37 ccm Wasser verwendet. Die Substanz wurde bei 55° gelöst, 10 Min. mit Aktivkohle behandelt und bei 55° filtriert. Danach wurde abgekühlt und bei 4° der Kristallisation überlassen. Ausb. 22 g (55 % d. Th.); Schmp. 56–58° (Hydrat) und 124° (wasserfrei). Leicht löslich in Äthanol, Dioxan, Aceton und Eisessig, wenig löslich in Wasser, Benzol, Chloroform und Essigester.  $[\alpha]_D^{20} = +1.30 \cdot 5/1 \cdot 0.0661 = +98.5^\circ$  (in Äthanol).

$C_{14}H_{20}O_8S$  (348.4) Ber. C 48.27 H 5.78 S 9.21 Gef. C 48.15 H 5.92 S 9.4

Die Perjodattitration<sup>36)</sup> ergab einen Verbrauch von 1.87 Moll. Perjodat pro Mol. Substanz.

2. *6-Methansulfonyl-methyl- $\alpha$ -D-glucosid (III) und dessen Triacetylderivat*: 12.0 g  *$\alpha$ -Methylglucosid* wurden in 100 ccm absol. Pyridin bei –20° unter Rühren langsam mit 5.0 ccm *Methansulfonsäurechlorid* versetzt. Nach 24 stdg. Aufbewahren bei 0° und weiteren 24 Stdn. bei 20° wurde i. Vak. bei 40° zum Sirup eingedampft, 2 mal in Propanol gelöst und bei 50° abdestilliert. Die Verbindung kristallisierte nicht. Der völlig trockene Sirup wurde in Pyridin gelöst, mit 24 ccm *Acetanhydrid* 24 Stdn. bei 20° acetyliert, das Lösungsmittel i. Vak. abgedampft und der Sirup in Chloroform aufgenommen. Nach Schütteln mit Kaliumhydrogensulfat und Natriumhydrogencarbonatlösung wurde das Chloroform entfernt und der völlig trockene Sirup im gleichen Vol. absol. Äthanol unter Rückfluß gelöst. Beim Abkühlen und Reiben kristallisierte die Substanz. Ausb. 16.5 g (67 % d. Th.); Schmp. 113° (aus Methanol).

3. *6-Tosyl-benzyl- $\beta$ -D-glucosid*: 31.5 g *Benzyl- $\beta$ -D-glucosid*, in 400 ccm absol. Pyridin gelöst, wurden bei –15° unter Schütteln mit 24.6 g *p*-Toluolsulfonsäurechlorid in 80 ccm Pyridin umgesetzt. Das Reaktionsgemisch wurde 30 Min. bei –15° und 12 Stdn. bei 20° gehalten, das Pyridin i. Vak. bei 40° abdestilliert, der Rückstand in Chloroform aufgenommen und

<sup>36)</sup> B. LYTHGOE und A. R. TODD, J. chem. Soc. [London] 1944, 592.

die chloroformische Lösung in der üblichen Weise gereinigt. Da die Lösung zur Gelbildung neigt, wurden alle Operationen bei 50° vorgenommen. Nach Vertreiben des Chloroforms wurde das trockene Pulver in 1.9/ Toluol unter Rückfluß gelöst und der Kristallisation überlassen. Ausb. 32 g (65 % d. Th.). Beim Umkristallisieren aus Wasser wurde auf 5 g Substanz 1/1 Wasser verwendet. Die Substanz kristallisierte als Hydrat, welches bei 55° i. Vak. 2 Moll. Kristallwasser verliert. Schmp. 116–117° (Zers.);  $[\alpha]_D^{20}$ :  $-0.24 \cdot 5/1 \cdot 0.0318 = -38^\circ$  (in Aceton).

$C_{20}H_{24}O_8S$  (424.5) Ber. C 56.58 H 5.69 S 7.56 Gef. C 56.64 H 5.87 S 7.45

4. *Hydrochlorid des 6-Desoxy-6-amino-methyl- $\alpha$ -D-glucosids (V)*: 6 g II wurden in 130 ccm absol. Methanol gelöst und bei 0° mit getrocknetem Ammoniak gesättigt. Dann wurde im Autoklaven 16 Stdn. auf 120° erwärmt, nach dem Erkalten der dunkle Inhalt mit Aktivkohle aufgeköcht, filtriert und die Lösung eingengt. Der schwach gelb gefärbte Sirup wurde in Wasser aufgenommen und unter Ausschluß von  $CO_2$  über eine Ionenaustauschersäule IRA 400 ( $OH^-$ -Form) gegeben. Das Eluat wurde unter Ausschluß von  $CO_2$  bei 40° zum Sirup eingedampft und dieser über  $P_4O_{10}$  getrocknet. Ausb. 2.5 g (75 % d. Th.). Dann wurde in 15 ccm absol. Methanol gelöst und so lange trockener Chlorwasserstoff eingeleitet, bis der durch Tüpfeln auf Indikatorpapier ermittelte  $pH$ -Wert ca. 5 beträgt. Beim Anreiben kristallisierte das salzsaure Salz des Aminozuckers, durch Zugabe von Äther wurde die Kristallisation vermehrt. Es wurde aus 50 ccm absol. Methanol mit Aktivkohle umkristallisiert und nach dem Erkalten 25 ccm absol. Äther hinzugefügt. Ausb. 2.5 g (64 % d. Th.); Schmp. 180 bis 200° (Zers.);  $[\alpha]_D^{20}$ :  $+1.42 \cdot 5/1 \cdot 0.0493 = +147^\circ$  (in Wasser).

$C_7H_{15}NO_5 \cdot HCl$  (229.7) Ber. C 36.58 H 7.02 N 6.10 Gef. C 36.55 H 7.13 N 5.81

Der Verlauf der Hydrolyse wurde papierchromatographisch und durch Methoxylbestimmung der Reaktionsprodukte verfolgt.

Konz. der zur Hydrolyse verwendeten Säure	Temp. °C	Stdn.	Gef. Methoxylwert in %	Umsetzungsgrad in %
0.2 n	100	8	13.0	—
1 n	100	2	10.15	—
1 n	100	4	6.5	25
1.5 n	100	3	2.8	50
2 n	100	4	0.97	80
				93

5. *Hydrochlorid des 6-Desoxy-6-amino-benzyl- $\beta$ -D-glucosids (VI)*: 11 g 6-Tosyl-benzyl- $\beta$ -D-glucosid wurden in 400 ccm Methanol, wie unter 4. beschrieben, mit Ammoniak behandelt. Der Autoklaveninhalt wurde mit Aktivkohle aufgeköcht, die Lösung eingengt, mit 250 ccm Methanol aufgenommen und erneut in der Wärme mit Aktivkohle behandelt. Der nach Abdampfen des Methanols zurückbleibende gelbe Sirup wurde in 250 ccm 50-proz. wäbr. Methanol aufgenommen und mit dem Ionenaustauscher behandelt. Anschließend wurde mit 300 ccm 50-proz. Methanol nachgewaschen und die Lösungen wie oben eingengt. Der zurückbleibende Sirup wurde in 15 ccm Methanol gelöst und wie oben Chlorwasserstoff eingeleitet. Nach Zugabe von 15 ccm Äther setzte die Kristallisation ein. Umkristallisiert wurde aus 15 ccm absol. Methanol und 15 ccm absol. Äther. Ausb. 4 g (51 % d. Th.); Schmp. 165°;  $[\alpha]_D^{20}$ :  $-0.31 \cdot 5/1 \cdot 0.0425 = -37^\circ$  (in Wasser).

$C_{13}H_{19}NO_5 \cdot HCl$  (305.9) Ber. C 51.03 H 6.39 N 4.57 Gef. C 50.88 H 6.44 N 4.33

6. *Hydrochlorid der 6-Desoxy-6-amino-D-glucose (VII)*: 3.6 g VI wurden in wäbr. Lösung mit 5-proz. Palladium-Tierkohle hydriert. Nach 4 Stdn. war die Reaktion beendet, die Lösung wurde filtriert und vorsichtig zum Sirup eingengt. Die Substanz kristallisierte nicht,

sie war chromatographisch rein und ergab sowohl mit Anilinphthalat wie auch mit Ninydrin eine Reaktion. Mit absol. Methanol verfestigte sich der Sirup zu einer amorphen Masse.

$C_6H_{13}NO_5 \cdot HCl$  (215.6) Ber. C 33.40 H 6.54 N 6.49

Gef. C 32.98 H 6.58 N 6.28 Asche 0.6

7. *6-Desoxy-6-azido-methyl- $\alpha$ -D-glucosid (VIII) und Triacetylderivat (IX)*: 3.48 g II wurden in 55 ccm Aceton gelöst und 2.6 g Natriumazid in 20 ccm Wasser hinzugefügt. Es wurde 72 Stdn. unter gelindem Sieden erhitzt und danach das Lösungsmittel i. Vak. abgedampft. Der Rückstand wurde in absol. Aceton aufgenommen, wobei die Salze ungelöst zurückbleiben, und das Aceton abgedampft. Der zurückbleibende Sirup wurde in 50 ccm Pyridin mit Acetanhydrid in der üblichen Weise acetyliert. Nach Abdampfen des Pyridins i. Vak. wurde in wenig siedendem Äthanol aufgenommen, filtriert und bei 4° zur Kristallisation gebracht. Ausb. 2.01 g (58 % d. Th.); Schmp. 103° (Äthanol).  $[\alpha]_D^{25}$ : +1.95° 10/1-0.1250 = +156° (in Methanol).

$C_{13}H_{19}N_3O_8$  (345.3) Ber. OCH<sub>3</sub> 8.99 N 12.17 Gef. OCH<sub>3</sub> 9.14 N 12.38

8. *1,2-Isopropyliden-6-desoxy-6-azido- $\alpha$ -D-glucofuranose (X)*: 6 g 1,2-Isopropyliden-6-mesyl-glucose und 7.5 g Natriumazid wurden in 50 ccm Aceton mit 35 ccm Wasser gelöst. Die klare Lösung wurde 66 Stdn. unter Rückfluß erwärmt, die Lösungsmittel i. Vak. abgedampft und der Rückstand gut getrocknet. Dann wurde mehrmals mit trockenem Aceton extrahiert, wobei die Salze ungelöst zurückblieben, und das Aceton i. Vak. entfernt. Dabei fiel das Produkt direkt krist. an. Es wurde aus Benzol umkristallisiert und zur vollständigen Abscheidung mit Ligroin versetzt. Ausb. 3.35 g (67 % d. Th.). Die Reaktion kann in gleicher Weise mit dem 6-Tosylderivat ausgeführt werden. Schmp. 104°;  $[\alpha]_D^{25}$ : +0.03° 10/1-0.1211 = +2.48° (in Aceton).

$C_9H_{15}N_3O_5$  (245.2) Ber. C 44.08 H 6.16 N 17.14 Gef. C 44.09 H 6.04 N 17.03

*Acetylderivat (XII)*: Die Azidoverbindung X ließ sich in der üblichen Weise mit Pyridin/Acetanhydrid acetylieren. Die 1,2-Isopropyliden-3,5-diacetyl-6-desoxy-6-azido-glucose (XII) ließ sich aus Methanol/Wasser (1:1) umkristallisieren. Ausb. 94 % d. Th. Schmp. 66–67°;  $[\alpha]_D^{25}$ : +0.14° 10/1-0.1499 = +9.3° (in Aceton).

$C_{13}H_{19}N_3O_7$  (329.3) Ber. C 47.41 H 5.82 N 12.76 Gef. C 47.60 H 5.82 N 12.97

9. *Hydrochlorid des 6-Desoxy-6-amino-methyl- $\alpha$ -D-glucosids (V) durch Hydrierung*: Das nach 7. dargestellte, sirupöse VIII wurde in 70 ccm Äthanol gelöst, 5 Spatelspitzen 5-proz. Palladium/Tierkohle hinzugefügt und durch die Mischung unter gleichzeitigem Schütteln mit einem Vibrator Wasserstoff hindurchgeleitet. Das Hydriergefäß wurde bei 60–70° gehalten. Nach 45 Min. gab man verd. Salzsäure bis zur schwach sauren Reaktion hinzu und leitete dann noch weitere 2 Stdn. Wasserstoff ein. Danach wurde vom Katalysator abfiltriert und i. Vak. eingedampft. Das zurückbleibende Hydrochlorid wurde in Methanol gelöst, mit Tierkohle behandelt und danach mit Äther zur Kristallisation gebracht. Ausb. 1.1 g (48 % d. Th., bez. auf II). Schmp. 195–200° (Zers.);  $[\alpha]_D^{25}$ : +147° (c = 1, in Wasser). Die Substanz ist identisch mit der nach 4. dargestellten.

$C_7H_{15}NO_5 \cdot HCl$  (229.6) Ber. OCH<sub>3</sub> 13.52 N 6.11 Gef. OCH<sub>3</sub> 13.84 N 5.94

*Acetylderivat*: 1 g der Acetylverbindung IX wurde in 100 ccm Methanol mit Raney-Nickel hydriert, wobei nach und nach mit verd. Salzsäure neutralisiert wurde (Tüpfeln!). Nach der üblichen Aufarbeitung wurde in absol. Methanol gelöst und mit Äther zur Kristallisation gebracht. Ausb. 0.3 g (30 % d. Th.).  $[\alpha]_D^{25}$ : +1.57° 5/1-0.0645 = +120.8° (in Wasser); Schmp. 200–210° (Zers.).

$C_{13}H_{21}NO_8 \cdot HCl$  (355.8) Ber. OCH<sub>3</sub> 8.72 N 3.94 Gef. OCH<sub>3</sub> 8.89 N 3.89

an asserted utility, unless countervailing evidence can be provided that shows that one of ordinary skill in the art would have a legitimate basis to doubt the credibility of such a statement. Similarly, Office personnel must accept an opinion from a qualified expert that is based upon relevant facts whose accuracy is not being questioned; it is improper to disregard the opinion solely because of a disagreement over the significance or meaning of the facts offered.

Once a *prima facie* showing of no specific and substantial credible utility has been properly established, the applicant bears the burden of rebutting it. The applicant can do this by amending the claims, by providing reasoning or arguments, or by providing evidence in the form of a declaration under 37 CFR 1.132 or a patent or a printed publication that rebuts the basis or logic of the *prima facie* showing. If the applicant responds to the *prima facie* rejection, the Office personnel should review the original disclosure, any evidence relied upon in establishing the *prima facie* showing, any claim amendments, and any new reasoning or evidence provided by the applicant in support of an asserted specific and substantial credible utility. It is essential for Office personnel to recognize, fully consider and respond to each substantive element of any response to a rejection based on lack of utility. Only where the totality of the record continues to show that the asserted utility is not specific, substantial, and credible should a rejection based on lack of utility be maintained.

If the applicant satisfactorily rebuts a *prima facie* rejection based on lack of utility under § 101, withdraw the § 101 rejection and the corresponding rejection imposed under § 112, first paragraph.

Dated: December 29, 2000.

Q. Todd Dickinson,

Under Secretary of Commerce for Intellectual Property and Director of the United States Patent and Trademark Office.

[FR Doc. 01-322 Filed 1-4-01; 8:45 am]

BILLING CODE 3510-16-U

## DEPARTMENT OF COMMERCE

### United States Patent and Trademark Office

[Docket No. 991027288-0264-02]

RIN 0651-AB10

### Guidelines for Examination of Patent Applications Under the 35 U.S.C. 112, ¶ 1, "Written Description" Requirement

AGENCY: United States Patent and Trademark Office, Commerce.

ACTION: Notice.

**SUMMARY:** These Guidelines will be used by USPTO personnel in their review of patent applications for compliance with the "written description" requirement of 35 U.S.C. 112, ¶ 1. These Guidelines supersede the "Revised Interim Guidelines for Examination of Patent Applications Under the 35 U.S.C. 112, ¶ 1 'Written Description' Requirement" that were published in the Federal Register at 64 FR 71427, Dec. 21, 1999, and in the Official Gazette at 1231 O.G. 123, Feb. 29, 2000. These Guidelines reflect the current understanding of the USPTO regarding the written description requirement of 35 U.S.C. 112, ¶ 1, and are applicable to all technologies.

**DATES:** The Guidelines are effective as of January 5, 2001.

**FOR FURTHER INFORMATION CONTACT:** Stephen Walsh by telephone at (703) 305-9035, by facsimile at (703) 305-9373, by mail to his attention addressed to United States Patent and Trademark Office, Box 8, Washington, DC 20231, or by electronic mail at "stephen.walsh@uspto.gov"; or Linda Therborn by telephone at (703) 305-8800, by facsimile at (703) 305-8825, by mail addressed to Box Comments, Commissioner for Patents, Washington, DC 20231, or by electronic mail at "linda.therborn@uspto.gov."

**SUPPLEMENTARY INFORMATION:** As of the publication date of this notice, these Guidelines will be used by USPTO personnel in their review of patent applications for compliance with the "written description" requirement of 35 U.S.C. 112, ¶ 1. Because these Guidelines only govern internal practices, they are exempt from notice and comment rulemaking under 5 U.S.C. 553(b)(A).

### Discussion of Public Comments

Comments were received from 48 individuals and 18 organizations in response to the request for comments on the "Revised Interim Guidelines for Examination of Patent Applications

Under the 35 U.S.C. 112, ¶ 1 'Written Description' Requirement" published in the Federal Register at 64 FR 71427, Dec. 21, 1999, and in the Official Gazette at 1231 O.G. 123, Feb. 29, 2000. The written comments have been carefully considered.

### Overview of Comments

The majority of comments favored issuance of final written description guidelines with minor revisions. Comments pertaining to the written description guidelines are addressed in detail below. A few comments addressed particular concerns with respect to the associated examiner training materials that are available for public inspection at the USPTO web site ([www.uspto.gov](http://www.uspto.gov)). Such comments will be taken under advisement in the revision of the training materials; consequently, these comments are not specifically addressed below as they do not impact the content of the Guidelines. Several comments raised issues pertaining to the patentability of ESTs, genes, or genomic inventions with respect to subject matter eligibility (35 U.S.C. 101), novelty (35 U.S.C. 102), or obviousness (35 U.S.C. 103). As these comments do not pertain to the written description requirement under 35 U.S.C. 112, they have not been addressed. However, the aforementioned comments are fully addressed in the "Discussion of Public Comments" in the "Utility Examination Guidelines" Final Notice, which will be published at or about the same time as the present Guidelines.

### Responses to Specific Comments

(1) *Comment:* One comment stated that the Guidelines instruct the patent examiner to determine the correspondence between what applicant has described as the essential identifying characteristic features of the invention and what applicant has claimed, and that such analysis will lead to error. According to the comment, the examiner may decide what applicant should have claimed and reject the claim for failure to claim what the examiner considers to be the invention. Another comment suggested that the Guidelines should clarify what is meant by "essential features of the invention." Another comment suggested that what applicant has identified as the "essential distinguishing characteristics" of the invention should be understood in terms of *Fiers v. Revel*, 984 F.2d 1164, 1169, 25 USPQ2d 1601, 1605 (Fed. Cir. 1993) ("Conception of a substance claimed *per se* without reference to a process requires conception of its structure, name,

formula, or definitive chemical or physical properties.").

*Response:* The suggestions have been adopted in part. The purpose of the written description analysis is to confirm that applicant had possession of what is claimed. The Guidelines have been modified to instruct the examiners to compare the scope of the invention claimed with the scope of what applicant has defined in the description of the invention. That is, the Guidelines instruct the examiner to look for consistency between a claim and what provides adequate factual support for the claim as judged by one of ordinary skill in the art from reading the corresponding written description.

(2) *Comment:* Two comments urge that *Regents of the University of California v. Eli Lilly & Co.*, 119 F.3d 1559, 43 USPQ2d 1398 (Fed. Cir. 1997), is bad law and should not be followed by the USPTO because it conflicts with binding precedent, such as *Vas-Cath v. Mahurkar*, 935 F.2d 1555, 19 USPQ2d 1111 (Fed. Cir. 1991). *Response:* The final Guidelines are based on the Office's current understanding of the law and are believed to be fully consistent with binding precedent of the U.S. Supreme Court and the U.S. Court of Appeals for the Federal Circuit. *Eli Lilly* is a precedential decision by the Court that has exclusive jurisdiction over appeals involving patent law. Accordingly, the USPTO must follow *Eli Lilly*. Furthermore, the USPTO does not view *Eli Lilly* as conflicting with *Vas-Cath*. *Vas-Cath* explains that the purpose of the written description requirement is to ensure that the applicant has conveyed to those of skill in the art that he or she was in possession of the claimed invention at the time of filing. *Vas-Cath*, 935 F.2d at 1563-64, 19 USPQ2d at 1117. *Eli Lilly* explains that a chemical compound's name does not necessarily convey a written description of the named chemical compound, particularly when a genus of compounds is claimed. *Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1405. The name, if it does no more than distinguish the claimed genus from all others by function, does not satisfy the written description requirement because "it does not define any structural features commonly possessed by members of the genus that distinguish them from others. One skilled in the art therefore cannot, as one can do with a fully described genus, visualize or recognize the identity of the members of the genus." *Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406. Thus, *Eli Lilly* identified a set of circumstances in which the words of the claim did not, without more, adequately convey to

others that applicants had possession of what they claimed.

(3) *Comment:* Several comments urged that the Guidelines do not recognize the inconsistency between the original claim doctrine and the written description requirement as set out in *Fiers* and *Eli Lilly*. On the other hand, another comment asserts that there is no strong presumption that an originally filed claim constitutes an adequate written description of the claimed subject matter. Several comments indicate that *in haec verba* support should be sufficient to comply with the written description requirement. Two comments urge that the concept of constructive reduction to practice upon filing of an application has been ignored. *Response:* As noted above, the USPTO does not find *Fiers* and *Eli Lilly* to be in conflict with binding precedent. An original claim may provide written description for itself, but it still must be an adequate written description which establishes that the inventor was in possession of the invention. The "original claim doctrine" is founded on cases which stand for the proposition that originally filed claims are part of the written description of an application as filed, and thus subject matter which is present only in originally filed claims need not find independent support in the specification. See, e.g., *In re Koller*, 613 F.2d 819, 824, 204 USPQ 702, 706 (CCPA 1980) (later added claims of similar scope and wording were adequately described by original claims); *In re Gardner*, 480 F.2d 879, 880, 178 USPQ 149, 149 (CCPA 1973) ("Under these circumstances, we consider the original claim in itself adequate 'written description' of the claimed invention. It was equally a 'written description' \* \* \* whether located among the original claims or in the descriptive part of the specification."). However, as noted in the preceding comment, *Eli Lilly* identified a set of circumstances in which the words of the claim did not, without more, adequately convey to others that applicants had possession of what they claimed. When the name of a novel chemical compound does not convey sufficient structural information about the compound to identify the compound, merely reciting the name is not enough to show that the inventor had possession of the compound at the time the name was written. The Guidelines indicate that there is a "strong presumption" that an adequate written description of the claimed invention is present when the application is filed, consistent with *In re Wertheim*, 541 F.2d 257, 263, 191 USPQ

90, 97 (CCPA 1976) ("we are of the opinion that the PTO has the initial burden of presenting evidence or reasons why persons skilled in the art would not recognize in the disclosure a description of the invention defined by the claims."). In most cases, the statement that "an originally filed claim is its own written description," is borne out because the claim language conveys to others of skill in the art that the applicant was "in possession" of what is claimed. The Guidelines emphasize that the burden of proof is on the examiner to establish that a description as filed is not adequate and require the examiner to introduce sufficient evidence or technical reasoning to shift the burden of going forward with contrary evidence to the applicant.

(4) *Comment:* One comment stated that the Guidelines change the substance of the written description requirement to require some level of enablement. The comment stated that the *Eli Lilly* case should not be followed because its change in the quality of the description required is in conflict with precedent. Another comment suggested that to comply with the written description requirement, the description must both (i) demonstrate possession of the claimed invention by the applicant; and (ii) put the public in possession of the claimed invention. *Response:* As noted in the comment above, the USPTO is bound by the Federal Circuit's decision in *Eli Lilly*. The Guidelines have been revised to clarify that an applicant must provide a description of the claimed invention which shows that applicant was in possession of the claimed invention. The suggestion to emphasize that the written description requirement must put the public in possession of the invention has not been adopted because it removes much of the distinction between the written description requirement and the enablement requirement. Although the two concepts are entwined, they are distinct and each is evaluated under separate legal criteria. The written description requirement, a question of fact, ensures that the inventor conveys to others that he or she had possession of the claimed invention; whereas, the enablement requirement, a question of law, ensures that the inventor conveys to others how to make and use the claimed invention.

(5) *Comment:* One comment suggested that the Guidelines should provide examples of situations in which the written description requirement was met but the enablement requirement was not, and vice versa. Another comment stated that examiners often use enablement language in making



written description rejections.

**Response:** The enablement and written description requirements are not coextensive and, therefore, situations will arise in which one requirement is met but the other is not. Federal Circuit case law demonstrates many circumstances where enablement or written description issues, but not both, were before the Court. These Guidelines are intended to clarify for the examining corps the criteria needed to satisfy the written description requirement. For examples applying these Guidelines to hypothetical fact situations, see the "Synopsis of Application of Written Description Guidelines" (examiner training materials available on-line at <http://www.uspto.gov/web/menu/written.pdf>). These examples, as well as the examination form paragraphs and instructions on their proper use, provide the appropriate language examiners should use in making written description rejections.

(6) **Comment:** One comment disagreed with the statement in an endnote that "the fact that a great deal more than just a process is necessary to render a product invention obvious means that a great deal more than just a process is necessary to provide written description for a product invention." The comment indicated that the statement is overly broad and inconsistent with the "strong presumption that an adequate written description of the claimed invention is present when the application is filed." As an extreme case, for example, for product-by-process claims, nothing else would be needed to provide the written description of the product. **Response:** The endnote has been clarified and is now more narrowly drawn. However, there is no *per se* rule that disclosure of a process is sufficient to adequately describe the products produced by the process. In fact, *Fiers v. Revel* and *Eli Lilly* involved special circumstances where the disclosure of a process of making and the function of the product alone did not provide an adequate written description for product claims. Even when a product is claimed in a product-by-process format, the adequacy of the written description of the process to support product claims must be evaluated on a case-by-case basis.

(7) **Comment:** Several comments urge that actual reduction to practice, as a method of satisfying the written description requirement by demonstrating possession, has been over-emphasized. **Response:** The Guidelines have been clarified to state that describing an actual reduction to practice is one of a number of ways to show possession of the invention.

Description of an actual reduction to practice offers an important "safe haven" that applies to all applications and is just one of several ways by which an applicant may demonstrate possession of the claimed invention. Actual reduction to practice may be crucial in the relatively rare instances where the level of knowledge and level of skill are such that those of skill in the art cannot describe a composition structurally, or specify a process of making a composition by naming components and combining steps, in such a way as to distinguish the composition with particularity from all others. Thus, the emphasis on actual reduction to practice is appropriate in those cases where the inventor cannot provide an adequate description of what the composition is, and a definition by function is insufficient to define a composition "because it is only an indication of what the [composition] does, rather than what it is." *Eli Lilly*, 119 F.3d at 1568, 43 USPQ at 1406. See also *Amgen Inc. v. Chugai Pharmaceutical Co.*, 927 F.2d 1200, 1206, 18 USPQ2d 1016, 1021 (Fed. Cir. 1991).

(8) **Comment:** One comment asserts that the citation to *Pfaff v. Wells Electronics, Inc.*, 525 U.S. 55, 48 USPQ2d 1641 (1998) is inappropriate and should be deleted because *Pfaff* is concerned with § 102(b) on-sale bar, not written description. Another comment suggested that the Guidelines should provide an explanation of how the "ready for patenting" concept of *Pfaff* should be used in determining compliance with the written description requirement. **Response:** The Guidelines state the general principle that actual reduction to practice is not required to show possession of, or to adequately describe, a claimed invention (although, as noted in the previous comment, an actual reduction to practice is crucial in relatively rare instances). An alternative is to show that the invention described was "ready for patenting" as set out in *Pfaff*. For example, a description of activities that demonstrates the invention was "ready for patenting" satisfies the written description requirement. As *Wertheim* indicates, "how the specification accomplishes this is not material." 541 F.2d at 262, 191 USPQ at 96.

(9) **Comment:** One comment stated that the written description of a claimed DNA should be required to include the complete sequence of the DNA and claims should be limited to the DNA sequence disclosed. **Response:** Describing the complete chemical structure, i.e., the DNA sequence, of a claimed DNA is one method of

satisfying the written description requirement, but it is not the only method. See *Eli Lilly*, 119 F.3d at 1566, 43 USPQ2d at 1404 ("An adequate written description of a DNA \* \* \* requires a precise definition, such as by structure, formula, chemical name, or physical properties." (emphasis added, internal quote omitted)). Therefore, there is no basis for a *per se* rule requiring disclosure of complete DNA sequences or limiting DNA claims to only the sequence disclosed.

(10) **Comment:** One comment stated that it is difficult to envision how one could provide a description of sufficient identifying characteristics of the invention without physical possession of a species of the invention, and thus this manner of showing possession should be considered as a way to show actual reduction to practice. **Response:** This suggestion has not been adopted. The three ways of demonstrating possession as set forth in the Guidelines are merely exemplary and are not mutually exclusive. While there are some cases where a description of sufficient relevant identifying characteristics will evidence an actual reduction to practice, there are other cases where it will not. See, e.g., *Ralston Purina Co. v. Far-Mar-Co, Inc.*, 772 F.2d 1570, 1576, 227 USPQ 177, 180 (Fed. Cir. 1985) (disclosure taken with the knowledge of those skilled in the art may be sufficient support for claims).

(11) **Comment:** One comment stated that the Guidelines should be revised to indicate that the test of disclosure of sufficiently detailed drawings should be expanded to include structural claiming of chemical entities. **Response:** The suggestion has been adopted.

(12) **Comment:** One comment stated that the Guidelines should reflect that an inventor is in possession of the invention when the inventor demonstrably has at least a complete conception thereof, and that factors and attributes which provide proof of written description should include evidence typically provided to prove a complete conception. **Response:** The suggestion has not been adopted because the conception analysis typically involves documentary evidence in addition to the description of the invention in the application as filed. However, it is acknowledged that if evidence typically provided to prove a complete conception is present in the specification as filed, it would be sufficient to show possession. The Federal Circuit has stated "[t]he conception analysis necessarily turns on the inventor's ability to describe his invention with particularity. Until he can do so, he cannot prove possession

of the complete mental picture of the invention." *Burroughs Wellcome Co. v. Barr Labs., Inc.*, 40 F.3d 1223, 1228, 32 USPQ2d 1915, 1919 (Fed. Cir. 1994). As further noted by the Federal Circuit, in order to prove conception, "a party must show possession of every feature recited in the count, and that every limitation of the count must have been known to the inventor at the time of the alleged conception." *Coleman v. Dines*, 754 F.2d 353, 359, 224 USPQ 857, 862 (Fed. Cir. 1985).

(13) *Comment*: One comment indicated that a "possession" test does not appear in Title 35 of the U.S. Code and is not clearly stated by the Federal Circuit. Therefore, it is recommended that patent examiners be directed to use existing judicial precedent to make rejections of claims unsupported by a statutory written description requirement. *Response*: While the Federal Circuit has not specifically laid out a "possession" test, the Court has clearly indicated that possession is a cornerstone of the written description inquiry. See, e.g., *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1563, 19 USPQ2d 1111, 1116 (Fed. Cir. 1991); see also *Purdue Pharma L.P. v. Faulding Inc.*, 230 F.3d 1320, 1323, 56 USPQ2d 1481, 1483 (Fed. Cir. 2000) ("[o]ne skilled in the art, reading the disclosure, must immediately discern the limitation at issue in the claims") (internal quote omitted). The possession test as set forth in the Guidelines is extrapolated from case law in a wide variety of technologies and is not intended to be limiting. Any rejections made by examiners will be made under 35 U.S.C. 112, ¶1, with supporting rationale. Final rejections are appealable if applicant disagrees and follows the required procedures to appeal.

(14) *Comment*: Two comments indicated that if the amino acid sequence for a polypeptide whose utility has been identified is described, then the question of possession of a class of nucleotides encoding that polypeptide can be addressed as a relatively routine matter using the understanding of the genetic code, and that the endnote addressing this issue should be revised. *Response*: The suggestion of these comments has been incorporated in the Guidelines and will be reflected in the training materials. However, based upon *In re Bell*, 991 F.2d 781, 785, 26 USPQ2d 1529, 1532 (Fed. Cir. 1993) and *In re Baird*, 16 F.3d 380, 382, 29 USPQ2d 1550, 1552 (Fed. Cir. 1994), this does not mean that applicant was in possession of any particular species of the broad genus.

(15) *Comment*: One comment disagreed with an endnote which stated

that a laundry list disclosure of moieties does not constitute a written description of every species in a genus. Specifically, the comment indicates that if the existence of a functional genus is adequately described in the specification, a laundry list of the species within that genus must satisfy the written description requirement.

*Response*: The suggestion to revise the endnote will not be adopted. A lack of adequate written description problem arises if the knowledge and level of skill in the art would not permit one skilled in the art to immediately envisage the product claimed from the disclosure. This was aptly demonstrated in *In re Bell* and *In re Baird* where possession of a large genus did not put a person of ordinary skill in the art in possession of any particular species. See also *Purdue Pharma*, 230 F.3d at 1328, 56 USPQ2d at 1487 (because the original specification did not disclose the later claimed concentration ratio was a part of the invention, the inventors cannot argue that they are merely narrowing a broad invention).

(16) *Comment*: One comment suggested that in the majority of cases, a single species will support a generic claim, and that the Guidelines should emphasize this point. *Response*: The suggestion has been adopted to a limited degree. The Guidelines now indicate that a single species may, in some instances, provide an adequate written description of a generic claim when the description of the species would evidence to one of ordinary skill in the art that the invention includes the genus. Note, however, *Tronzo v. Biomet, Inc.*, 156 F.3d 1154, 47 USPQ2d 1829 (Fed. Cir. 1998), where the species in the parent application was held not to provide written description support for the genus in the child application.

(17) *Comment*: One comment asserted that the Guidelines should focus on the compliance of the claims, not the specification, with the written description requirement. *Response*: This suggestion will not be adopted. "The specification shall contain a written description of the invention." 35 U.S.C. 112. The claims are part of the specification. *Id.*, ¶ 2. If an adequate description is provided, it will suffice "whether located among the original claims or in the descriptive part of the specification." *In re Gardner*, 480 F.2d 879, 880, 178 USPQ 149 (CCPA 1973). The entire disclosure, including the specification, drawings, and claims, must be considered.

(18) *Comment*: One comment asserted that the Guidelines confuse "new matter," 35 U.S.C. 132, with the written description requirement, and that the

same standard for written description should be applied to both original claims and new or amended claims. *Response*: The Guidelines indicate that for both original and amended claims, the inquiry is whether one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention at the time the application was filed.

(19) *Comment*: One comment suggested that the second paragraph of the section pertaining to determining what the claim as a whole covers should be deleted because it relates more to compliance with § 112, second paragraph, than with the written description requirement. *Response*: This suggestion will not be adopted. The claims must be construed and all issues as to the scope and meaning of the claim must be explored during the inquiry into whether the written description requirement has been met. The concept of treating the claim as a whole is applicable to all criteria for patentability.

(20) *Comment*: One comment suggested a different order for the general analysis for determining compliance with the written description requirement, starting with reading the claim, then the specification, and then determining whether the disclosure demonstrates possession by the applicant. *Response*: This suggestion will not be adopted. The claims must be construed as broadly as reasonable in light of the specification and the knowledge in the art. See *In re Morris*, 127 F.3d 1048, 1054, 44 USPQ2d 1023, 1027 (Fed. Cir. 1997). Then the disclosure must be evaluated to determine whether it adequately describes the claimed invention, i.e., whether it conveys to a person having ordinary skill in the art that the applicant had possession of what he or she now claims.

(21) *Comment*: Several comments suggested that the Guidelines are unclear with regard to how the examiner should treat the transitional phrase "consisting essentially of." The comments also suggested that the endnote that explains "consisting essentially of" does not make clear how the use of this intermediate transitional language affects the scope of the claim. Several comments stated that the USPTO does not have legal authority to treat claims reciting this language as open (equivalent to "comprising"). Another comment suggested that the phrase "clear indication in the specification" be replaced with "explicit or implicit indication." *Response*: The transitional phrase "consisting essentially of" "excludes

ingredients that would 'materially affect the basic and novel characteristics' of the claimed composition." *Atlas Powder Co. v. E.I. DuPont de Nemours & Co.*, 750 F.2d 1569, 1574, 224 USPQ 409, 412 (Fed. Cir. 1984). The basic and novel characteristics of the claimed invention are limited by the balance of the claim. *In re Janakirama-Rao*, 317 F.2d 951, 954, 137 USPQ 893, 896 (CCPA 1963). However, during prosecution claims must be read broadly, consistent with the specification. *In re Morris*, 127 F.3d 1048, 1054, 44 USPQ2d 1023, 1027 (Fed. Cir. 1997). Thus, for purposes of searching for and applying prior art in a rejection under 35 U.S.C. 102 or 103, if the specification or the claims do not define the "basic and novel" properties of the claimed subject matter (or if such properties are in dispute), the broadest reasonable interpretation consistent with the specification is that the basic and novel characteristics are merely the presence of the recited limitations. See, e.g., *Janakirama-Rao*, 317 F.2d at 954, 137 USPQ at 895-96. This does not indicate that the intermediate transitional language is never given weight. Applicants may amend the claims to avoid the rejections or seek to establish that the specification provides definitions of terms in the claims that define the basic and novel characteristics of the claimed invention which distinguish the claimed invention from the prior art. When an applicant contends that additional steps or materials in the prior art are excluded by the recitation of 'consisting essentially of,' applicant has the burden of showing that the introduction of additional steps or components would materially change the characteristics of applicant's invention. *In re De Lajarte*, 337 F.2d 870, 143 USPQ 256 (CCPA 1964). The language used in the Guidelines is consistent with *PPG Industries Inc. v. Guardian Industries Corp.*, 156 F.3d 1351, 1355, 48 USPQ2d 1351, 1355 (Fed. Cir. 1998) ("PPG could have defined the scope of the phrase 'consisting essentially of' for purposes of its patent by making clear in its specification what it regarded as constituting a material change in the basic and novel characteristics.").

(22) *Comment*: One comment stated that the written description should "disclose the invention," including why the invention works and how it was developed. *Response*: This suggestion has not been adopted. An inventor does not need to know how or why the invention works in order to obtain a patent. *Newman v. Quigg*, 877 F.2d 1575, 1581, 11 USPQ2d 1340, 1345

(Fed. Cir. 1989). To satisfy the enablement requirement of 35 U.S.C. 112, §1, an application must disclose the claimed invention in sufficient detail to enable a person of ordinary skill in the art to make and use the claimed invention. To satisfy the written description requirement of 35 U.S.C. 112, §1, the description must show that the applicant was in possession of the claimed invention at the time of filing. There is no statutory basis to require disclosure of why an invention works or how it was developed. "Patentability shall not be negated by the manner in which the invention was made." 35 U.S.C. 103(a).

(23) *Comment*: One comment recommended that the phrases "emerging and unpredictable technologies" and "unpredictable art" be replaced with the phrase—inventions characterized by factors which are not reasonably predictable in terms of the ordinary skill in the art—. *Response*: The suggestion is adopted in part and the recommended phrase has been added as an alternative.

(24) *Comment*: One comment recommended that the phrase "conventional in the art" be replaced with—part of the knowledge of one of ordinary skill in the art—. *Response*: The suggestion is adopted in part and the recommended phrase has been added as an alternative. The standard of "conventional in the art" is supported by case law holding that a patent specification "need not teach, and preferably omits, what is well known in the art." See *Spectra-Physics, Inc. v. Coherent, Inc.*, 827 F.2d 1524, 1534, 3 USPQ2d 1737, 1743 (Fed. Cir. 1987); *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986). See also *Atmel Corp. v. Information Storage Devices, Inc.*, 198 F.3d 1374, 1382, 53 USPQ2d 1225, 1231 (Fed. Cir. 1999).

(25) *Comment*: One comment recommended that the Guidelines be amended to state that the appropriate skill level for determining possession of the claimed invention is that of a person of ordinary skill in the art. *Response*: The comment has not been adopted. The statutory language itself indicates that compliance with the requirements of 35 U.S.C. 112, §1, is judged from the standard of "any person skilled in the art." It is noted, however, that the phrases "one of skill in the art" and "one of ordinary skill in the art" appear to be synonymous. See, e.g., *Union Oil Co. v. Atlantic Richfield Co.*, 208 F.3d 989, 997, 54 USPQ2d 1227, 1232 (Fed. Cir. 2000) ("The written description requirement does not require the applicant 'to describe exactly the subject

matter claimed, [instead] the description must clearly allow persons of ordinary skill in the art to recognize that [he or she] invented what is claimed." Thus, § 112, § 1, ensures that, as of the filing date, the inventor conveyed with reasonable clarity to those of skill in the art that he was in possession of the subject matter of the claims." (citations omitted, emphasis added)).

(26) *Comment*: One comment stated that an endnote misstates the relevant law in stating that, to show inherent written descriptive support for a claim limitation, the inherent disclosure must be such as would be recognized by a person of ordinary skill in the art. The comment recommended that the endnote be amended to delete the reference to recognition by persons of ordinary skill and to cite *Pingree v. Hull*, 518 F.2d 624, 186 USPQ 248 (CCPA 1975), rather than *In re Robertson*, 169 F.3d 743, 49 USPQ2d 1949 (Fed. Cir. 1999). *Response*: The comment has not been adopted. Federal Circuit precedent makes clear that an inherent disclosure must be recognized by those of ordinary skill in the art. See, e.g., *Hyatt v. Boone*, 146 F.3d 1348, 1354-55, 47 USPQ2d 1128, 1132 (Fed. Cir. 1998) ("[T]he purpose of the description requirement is 'to ensure that the inventor had possession, as of the filing date of the application relied on, of the specific subject matter later claimed by him.' \* \* \* Thus, the written description must include all of the limitations of the interference count, or the applicant must show that any absent text is necessarily comprehended in the description provided and would have been so understood at the time the patent application was filed." (emphasis added)). See also *Reiffin v. Microsoft Corp.*, 214 F.3d 1342, 1346, 54 USPQ2d 1915, 1917 (Fed. Cir. 2000) (The "application considered as a whole must convey to one of ordinary skill in the art, either explicitly or inherently, that [the inventor] invented the subject matter claimed \* \* \*." See \* \* \* *Continental Can Co. USA v. Monsanto Co.*, 948 F.2d 1264, 1268, 20 USPQ2d 1746, 1749 (Fed. Cir. 1991) (descriptive matter may be inherently present in a specification if one skilled in the art would necessarily recognize such a disclosure)").

(27) *Comment*: Several comments pointed out an inconsistency in the Federal Register Notice re: the Revised Interim Written Description Guidelines. The inconsistency concerned the treatment of claims directed to an isolated DNA comprising SEQ ID NO:1 wherein SEQ ID NO:1 is an expressed sequence tag. The comments contrasted paragraphs 34 and 35 of the Response to

Public Comments with the statement in the text of the Guidelines that a genus must be supported by a representative number of species (as analyzed in Example 7 of the training materials).

**Response:** The USPTO acknowledges that there was an inconsistency. The Office notes that a claim reciting a nucleic acid comprising SEQ ID NO:1 may be subject to a rejection for lack of an adequate written description where particular identifiable species within the scope of the claim lack an adequate written description. The training materials as amended exemplify an appropriate analysis.

(28) **Comment:** One comment stated that the USPTO should respond to the issue of whether the U.S. is meeting its TRIPs obligations. This comment noted that the USPTO did not address an earlier comment regarding the "Interim Guidelines for the Examination of Patent Applications under the 35 U.S.C. 112, § 1, 'Written Description' Requirement," 63 FR 32,639, June 15, 1998, which questioned whether the written description requirement is truly different from the enablement requirement, and indicated that such a requirement may be contrary to the TRIPs provisions of the World Trade Organization (Article 27.1). Article 27.1 requires WTO Members to, *inter alia*, make patents available, with limited exceptions, for products and processes in all fields of technology so long as those products and processes are new, involve an inventive step, and are capable of industrial application. The comment further suggested a response. **Response:** TRIPs Article 27 does not address what must be included in a patent application to allow WTO Member officials to determine whether particular inventions meet the standards for patentability established in that Article. TRIPs Article 29, which is more relevant to this comment, states that Members "shall require" patent applicants to disclose their invention "in a manner sufficiently clear and complete for the invention to be carried out by a person skilled in the art." If the written description is not clear and complete, the applicant may not have been in possession of the invention. This may support both written description and enablement standards. In addition, Article 29 expressly authorizes Members to require patent applicants to disclose the best method the inventor knows at the time of filing an application for carrying out the invention.

(29) **Comment:** Two comments commended the USPTO for eliminating the Biotechnology Specific Examples in the Revised Interim Written Description

Guidelines and providing separate training materials. One comment indicated a need to reconfirm the examples set forth in the Interim Written Description Guidelines published in 1998. **Response:** The current training materials reflect the manner in which the USPTO interprets the Written Description Guidelines.

(30) **Comment:** Several comments addressed specific concerns about the examiner training materials. **Response:** The comments received with respect to the training materials will be taken under advisement as the Office revises the training materials in view of the revisions to the Guidelines. The specific comments will not be addressed herein as they do not impact the language of the Guidelines.

#### Guidelines for the Examination of Patent Applications Under the 35 U.S.C. 112, § 1, "Written Description" Requirement

These "Written Description Guidelines" are intended to assist Office personnel in the examination of patent applications for compliance with the written description requirement of 35 U.S.C. 112, § 1. This revision is based on the Office's current understanding of the law and public comments received in response to the USPTO's previous request for public comments on its Revised Interim Written Description Guidelines and is believed to be fully consistent with binding precedent of the U.S. Supreme Court, as well as the U.S. Court of Appeals for the Federal Circuit and its predecessor courts.

This revision does not constitute substantive rulemaking and hence does not have the force and effect of law. It is designed to assist Office personnel in analyzing claimed subject matter for compliance with substantive law. Rejections will be based upon the substantive law, and it is these rejections which are appealable. Consequently, any perceived failure by Office personnel to follow these Guidelines is neither appealable nor petitionable.

These Guidelines are intended to form part of the normal examination process. Thus, where Office personnel establish a *prima facie* case of lack of written description for a claim, a thorough review of the prior art and examination on the merits for compliance with the other statutory requirements, including those of 35 U.S.C. 101, 102, 103, and 112, is to be conducted prior to completing an Office action which includes a rejection for lack of written description. Office personnel are to rely on this revision of the Guidelines in the event of any inconsistent treatment of

issues involving the written description requirement between these Guidelines and any earlier guidance provided from the Office.

#### I. General Principles Governing Compliance With the "Written Description" Requirement for Applications

The first paragraph of 35 U.S.C. 112 requires that the "specification shall contain a written description of the invention \* \* \*." This requirement is separate and distinct from the enablement requirement.<sup>1</sup> The written description requirement has several policy objectives. "[T]he 'essential goal' of the description of the invention requirement is to clearly convey the information that an applicant has invented the subject matter which is claimed."<sup>2</sup> Another objective is to put the public in possession of what the applicant claims as the invention.<sup>3</sup> The written description requirement of the Patent Act promotes the progress of the useful arts by ensuring that patentees adequately describe their inventions in their patent specifications in exchange for the right to exclude others from practicing the invention for the duration of the patent's term.

To satisfy the written description requirement, a patent specification must describe the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention.<sup>4</sup> An applicant shows possession of the claimed invention by describing the claimed invention with all of its limitations using such descriptive means as words, structures, figures, diagrams, and formulas that fully set forth the claimed invention.<sup>5</sup> Possession may be shown in a variety of ways including description of an actual reduction to practice,<sup>6</sup> or by showing that the invention was "ready for patenting" such as by the disclosure of drawings or structural chemical formulas that show that the invention was complete,<sup>7</sup> or by describing distinguishing identifying characteristics sufficient to show that the applicant was in possession of the claimed invention.<sup>8</sup> A question as to whether a specification provides an adequate written description may arise in the context of an original claim which is not described sufficiently, a new or amended claim wherein a claim limitation has been added or removed, or a claim to entitlement of an earlier priority date or effective filing date under 35 U.S.C. 119, 120, or 365(c).<sup>9</sup> Compliance with the written description requirement is a question of

fact which must be resolved on a case-by-case basis.<sup>10</sup>

#### A. Original Claims

There is a strong presumption that an adequate written description of the claimed invention is present when the application is filed.<sup>11</sup> However, the issue of a lack of adequate written description may arise even for an original claim when an aspect of the claimed invention has not been described with sufficient particularity such that one skilled in the art would recognize that the applicant had possession of the claimed invention.<sup>12</sup> The claimed invention as a whole may not be adequately described if the claims require an essential or critical feature which is not adequately described in the specification and which is not conventional in the art or known to one of ordinary skill in the art.<sup>13</sup> This problem may arise where an invention is described solely in terms of a method of its making coupled with its function and there is no described or art-recognized correlation or relationship between the structure of the invention and its function.<sup>14</sup> A lack of adequate written description issue also arises if the knowledge and level of skill in the art would not permit one skilled in the art to immediately envisage the product claimed from the disclosed process.<sup>15</sup>

#### B. New or Amended Claims

The proscription against the introduction of new matter in a patent application<sup>16</sup> serves to prevent an applicant from adding information that goes beyond the subject matter originally filed.<sup>17</sup> Thus, the written description requirement prevents an applicant from claiming subject matter that was not adequately described in the specification as filed. New or amended claims which introduce elements or limitations which are not supported by the as-filed disclosure violate the written description requirement.<sup>18</sup> While there is no *in haec verba* requirement, newly added claim limitations must be supported in the specification through express, implicit, or inherent disclosure. An amendment to correct an obvious error does not constitute new matter where one skilled in the art would not only recognize the existence of the error in the specification, but also recognize the appropriate correction.<sup>19</sup> Deposits made after the application filing date cannot be relied upon to support additions to or correction of information in the application as filed.<sup>20</sup>

Under certain circumstances, omission of a limitation can raise an

issue regarding whether the inventor had possession of a broader, more generic invention.<sup>21</sup> A claim that omits an element which applicant describes as an essential or critical feature of the invention originally disclosed does not comply with the written description requirement.<sup>22</sup>

The fundamental factual inquiry is whether the specification conveys with reasonable clarity to those skilled in the art that, as of the filing date sought, applicant was in possession of the invention as now claimed.<sup>23</sup>

## II. Methodology for Determining Adequacy of Written Description

### A. Read and Analyze the Specification for Compliance With 35 U.S.C. 112, § 1

Office personnel should adhere to the following procedures when reviewing patent applications for compliance with the written description requirement of 35 U.S.C. 112, § 1. The examiner has the initial burden, after a thorough reading and evaluation of the content of the application, of presenting evidence or reasons why a person skilled in the art would not recognize that the written description of the invention provides support for the claims. There is a strong presumption that an adequate written description of the claimed invention is present in the specification as filed;<sup>24</sup> however, with respect to newly added or amended claims, applicant should show support in the original disclosure for the new or amended claims.<sup>25</sup> Consequently, rejection of an original claim for lack of written description should be rare. The inquiry into whether the description requirement is met is a question of fact that must be determined on a case-by-case basis.<sup>26</sup>

#### 1. For Each Claim, Determine What the Claim as a Whole Covers

Claim construction is an essential part of the examination process. Each claim must be separately analyzed and given its broadest reasonable interpretation in light of and consistent with the written description.<sup>27</sup> The entire claim must be considered, including the preamble language<sup>28</sup> and the transitional phrase.<sup>29</sup> The claim as a whole, including all limitations found in the preamble,<sup>30</sup> the transitional phrase, and the body of the claim, must be sufficiently supported to satisfy the written description requirement.<sup>31</sup>

The examiner should evaluate each claim to determine if sufficient structures, acts, or functions are recited to make clear the scope and meaning of the claim, including the weight to be given the preamble.<sup>32</sup> The absence of definitions or details for well-

established terms or procedures should not be the basis of a rejection under 35 U.S.C. 112, § 1, for lack of adequate written description. Limitations may not, however, be imported into the claims from the specification.

#### 2. Review the Entire Application to Understand How Applicant Provides Support for the Claimed Invention Including Each Element and/or Step

Prior to determining whether the disclosure satisfies the written description requirement for the claimed subject matter, the examiner should review the claims and the entire specification, including the specific embodiments, figures, and sequence listings, to understand how applicant provides support for the various features of the claimed invention.<sup>33</sup> The analysis of whether the specification complies with the written description requirement calls for the examiner to compare the scope of the claim with the scope of the description to determine whether applicant has demonstrated possession of the claimed invention. Such a review is conducted from the standpoint of one of skill in the art at the time the application was filed<sup>34</sup> and should include a determination of the field of the invention and the level of skill and knowledge in the art. Generally, there is an inverse correlation between the level of skill and knowledge in the art and the specificity of disclosure necessary to satisfy the written description requirement. Information which is well known in the art need not be described in detail in the specification.<sup>35</sup>

#### 3. Determine Whether There is Sufficient Written Description to Inform a Skilled Artisan That Applicant was in Possession of the Claimed Invention as a Whole at the Time the Application Was Filed

a. Original claims. Possession may be shown in many ways. For example, possession may be shown, *inter alia*, by describing an actual reduction to practice of the claimed invention. Possession may also be shown by a clear depiction of the invention in detailed drawings or in structural chemical formulas which permit a person skilled in the art to clearly recognize that applicant had possession of the claimed invention. An adequate written description of the invention may be shown by any description of sufficient, relevant, identifying characteristics so long as a person skilled in the art would recognize that the inventor had possession of the claimed invention.<sup>36</sup>

A specification may describe an actual reduction to practice by showing

that the inventor constructed an embodiment or performed a process that met all the limitations of the claim and determined that the invention would work for its intended purpose.<sup>37</sup> Description of an actual reduction to practice of a biological material may be shown by specifically describing a deposit made in accordance with the requirements of 37 CFR 1.801 *et seq.*<sup>38</sup>

An applicant may show possession of an invention by disclosure of drawings<sup>39</sup> or structural chemical formulas<sup>40</sup> that are sufficiently detailed to show that applicant was in possession of the claimed invention as a whole. The description need only describe in detail that which is new or not conventional.<sup>41</sup> This is equally true whether the claimed invention is directed to a product or a process.

An applicant may also show that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics<sup>42</sup> which provide evidence that applicant was in possession of the claimed invention,<sup>43</sup> *i.e.*, complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics.<sup>44</sup> What is conventional or well known to one of ordinary skill in the art need not be disclosed in detail.<sup>45</sup> If a skilled artisan would have understood the inventor to be in possession of the claimed invention at the time of filing, even if every nuance of the claims is not explicitly described in the specification, then the adequate description requirement is met.<sup>46</sup>

(1) For each claim drawn to a single embodiment or species:<sup>47</sup>

(a) Determine whether the application describes an actual reduction to practice of the claimed invention.

(b) If the application does not describe an actual reduction to practice, determine whether the invention is complete as evidenced by a reduction to drawings or structural chemical formulas that are sufficiently detailed to show that applicant was in possession of the claimed invention as a whole.

(c) If the application does not describe an actual reduction to practice or reduction to drawings or structural chemical formula as discussed above, determine whether the invention has been set forth in terms of distinguishing identifying characteristics as evidenced by other descriptions of the invention that are sufficiently detailed to show that applicant was in possession of the claimed invention.

(i) Determine whether the application as filed describes the complete structure

(or acts of a process) of the claimed invention as a whole. The complete structure of a species or embodiment typically satisfies the requirement that the description be set forth "in such full, clear, concise, and exact terms" to show possession of the claimed invention.<sup>48</sup> If a complete structure is disclosed, the written description requirement is satisfied for that species or embodiment, and a rejection under 35 U.S.C. 112, § 1, for lack of written description must not be made.

(ii) If the application as filed does not disclose the complete structure (or acts of a process) of the claimed invention as a whole, determine whether the specification discloses other relevant identifying characteristics sufficient to describe the claimed invention in such full, clear, concise, and exact terms that a skilled artisan would recognize applicant was in possession of the claimed invention.<sup>49</sup>

Whether the specification shows that applicant was in possession of the claimed invention is not a single, simple determination, but rather is a factual determination reached by considering a number of factors. Factors to be considered in determining whether there is sufficient evidence of possession include the level of skill and knowledge in the art, partial structure, physical and/or chemical properties, functional characteristics alone or coupled with a known or disclosed correlation between structure and function, and the method of making the claimed invention. Disclosure of any combination of such identifying characteristics that distinguish the claimed invention from other materials and would lead one of skill in the art to the conclusion that the applicant was in possession of the claimed species is sufficient.<sup>50</sup> Patents and printed publications in the art should be relied upon to determine whether an art is mature and what the level of knowledge and skill is in the art. In most technologies which are mature, and wherein the knowledge and level of skill in the art is high, a written description question should not be raised for original claims even if the specification discloses only a method of making the invention and the function of the invention.<sup>51</sup> In contrast, for inventions in emerging and unpredictable technologies, or for inventions characterized by factors not reasonably predictable which are known to one of ordinary skill in the art, more evidence is required to show possession. For example, disclosure of only a method of making the invention and the function may not be sufficient to support a product claim other than a

product-by-process claim.<sup>52</sup>

Furthermore, disclosure of a partial structure without additional characterization of the product may not be sufficient to evidence possession of the claimed invention.<sup>53</sup>

Any claim to a species that does not meet the test described under at least one of (a), (b), or (c) must be rejected as lacking adequate written description under 35 U.S.C. 112, § 1.

(2) For each claim drawn to a genus:

The written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species by actual reduction to practice (see (1)(a), above), reduction to drawings (see (1)(b), above), or by disclosure of relevant, identifying characteristics, *i.e.*, structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics, sufficient to show the applicant was in possession of the claimed genus (see (1)(c), above).<sup>54</sup>

A "representative number of species" means that the species which are adequately described are representative of the entire genus. Thus, when there is substantial variation within the genus, one must describe a sufficient variety of species to reflect the variation within the genus. On the other hand, there may be situations where one species adequately supports a genus.<sup>55</sup> What constitutes a "representative number" is an inverse function of the skill and knowledge in the art. Satisfactory disclosure of a "representative number" depends on whether one of skill in the art would recognize that the applicant was in possession of the necessary common attributes or features of the elements possessed by the members of the genus in view of the species disclosed. For inventions in an unpredictable art, adequate written description of a genus which embraces widely variant species *cannot* be achieved by disclosing only one species within the genus.<sup>56</sup> Description of a representative number of species does not require the description to be of such specificity that it would provide individual support for each species that the genus embraces.<sup>57</sup> If a representative number of adequately described species are not disclosed for a genus, the claim to that genus must be rejected as lacking adequate written description under 35 U.S.C. 112, § 1.

b. New claims, amended claims, or claims asserting entitlement to the benefit of an earlier priority date or filing date under 35 U.S.C. 119, 120, or



365(c). The examiner has the initial burden of presenting evidence or reasoning to explain why persons skilled in the art would not recognize in the original disclosure a description of the invention defined by the claims.<sup>58</sup> However, when filing an amendment an applicant should show support in the original disclosure for new or amended claims.<sup>59</sup> To comply with the written description requirement of 35 U.S.C. 112, § 1, or to be entitled to an earlier priority date or filing date under 35 U.S.C. 119, 120, or 365(c), each claim limitation must be expressly,<sup>60</sup> implicitly,<sup>61</sup> or inherently<sup>62</sup> supported in the originally filed disclosure.<sup>63</sup> Furthermore, each claim must include all elements which applicant has described as essential.<sup>64</sup>

If the originally filed disclosure does not provide support for each claim limitation, or if an element which applicant describes as essential or critical is not claimed, a new or amended claim must be rejected under 35 U.S.C. 112, § 1, as lacking adequate written description, or in the case of a claim for priority under 35 U.S.C. 119, 120, or 365(c), the claim for priority must be denied.

### III. Complete Patentability Determination Under All Statutory Requirements and Clearly Communicate Findings, Conclusions, and Their Bases

The above only describes how to determine whether the written description requirement of 35 U.S.C. 112, § 1, is satisfied. Regardless of the outcome of that determination, Office personnel must complete the patentability determination under all the relevant statutory provisions of title 35 of the U.S. Code.

Once Office personnel have concluded analysis of the claimed invention under all the statutory provisions, including 35 U.S.C. 101, 112, 102, and 103, they should review all the proposed rejections and their bases to confirm their correctness. Only then should any rejection be imposed in an Office action. The Office action should clearly communicate the findings, conclusions, and reasons which support them. When possible, the Office action should offer helpful suggestions on how to overcome rejections.

#### A. For Each Claim Lacking Written Description Support, Reject the Claim Under Section 112, § 1, for Lack of Adequate Written Description

A description as filed is presumed to be adequate, unless or until sufficient evidence or reasoning to the contrary

has been presented by the examiner to rebut the presumption.<sup>65</sup> The examiner, therefore, must have a reasonable basis to challenge the adequacy of the written description. The examiner has the initial burden of presenting by a preponderance of evidence why a person skilled in the art would not recognize in an applicant's disclosure a description of the invention defined by the claims.<sup>66</sup> In rejecting a claim, the examiner must set forth express findings of fact regarding the above analysis which support the lack of written description conclusion. These findings should:

(1) Identify the claim limitation at issue; and

(2) Establish a *prima facie* case by providing reasons why a person skilled in the art at the time the application was filed would not have recognized that the inventor was in possession of the invention as claimed in view of the disclosure of the application as filed. A general allegation of "unpredictability in the art" is not a sufficient reason to support a rejection for lack of adequate written description.

When appropriate, suggest amendments to the claims which can be supported by the application's written description, being mindful of the prohibition against the addition of new matter in the claims or description.<sup>67</sup>

#### B. Upon Reply by Applicant, Again Determine the Patentability of the Claimed Invention, Including Whether the Written Description Requirement Is Satisfied by Reperforming the Analysis Described Above in View of the Whole Record

Upon reply by applicant, before repeating any rejection under 35 U.S.C. 112, § 1, for lack of written description, review the basis for the rejection in view of the record as a whole, including amendments, arguments, and any evidence submitted by applicant. If the whole record now demonstrates that the written description requirement is satisfied, do not repeat the rejection in the next Office action. If the record still does not demonstrate that the written description is adequate to support the claim(s), repeat the rejection under 35 U.S.C. 112, § 1, fully respond to applicant's rebuttal arguments, and properly treat any further showings submitted by applicant in the reply. When a rejection is maintained, any affidavits relevant to the 112, § 1, written description requirement,<sup>68</sup> must be thoroughly analyzed and discussed in the next Office action.

Dated: December 29, 2000.

Q. Todd Dickinson,  
Under Secretary of Commerce for Intellectual Property and Director of the United States Patent and Trademark Office.

#### Endnotes

<sup>1</sup> See, e.g., *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1560, 19 USPQ2d 1111, 1114 (Fed. Cir. 1991).

<sup>2</sup> *In re Barker*, 559 F.2d 588, 592 n.4, 194 USPQ 470, 473 n.4 (CCPA 1977).

<sup>3</sup> See *Regents of the University of California v. Eli Lilly*, 119 F.3d 1559, 1566, 43 USPQ2d 1398, 1404 (Fed. Cir. 1997), cert. denied, 523 U.S. 1089 (1998).

<sup>4</sup> See, e.g., *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d at 1563, 19 USPQ2d at 1116. Much of the written description case law addresses whether the specification as originally filed supports claims not originally in the application. The issue raised in the cases is most often phrased as whether the original application provides "adequate support" for the claims at issue or whether the material added to the specification incorporates "new matter" in violation of 35 U.S.C. 132. The "written description" question similarly arises in the interference context, where the issue is whether the specification of one party to the interference can support the newly added claims corresponding to the count at issue, i.e., whether that party can "make the claim" corresponding to the interference count. See, e.g., *Martin v. Mayer*, 823 F.2d 500, 503, 3 USPQ2d 1333, 1335 (Fed. Cir. 1987).

In addition, early opinions suggest the Patent and Trademark Office was unwilling to find written descriptive support when the only description was found in the claims; however, this viewpoint was rejected. See *In re Koller*, 613 F.2d 819, 204 USPQ 702 (CCPA 1980) (original claims constitute their own description); accord *In re Gardner*, 475 F.2d 1389, 177 USPQ 396 (CCPA 1973); accord *In re Wertheim*, 541 F.2d 257, 191 USPQ 90 (CCPA 1976) (accord). It is now well accepted that a satisfactory description may be in the claims or any other portion of the originally filed specification. These early opinions did not address the quality or specificity of particularity that was required in the description, i.e., how much description is enough.

<sup>5</sup> *Lockwood v. American Airlines, Inc.*, 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997).

<sup>6</sup> An application specification may show actual reduction to practice by describing testing of the claimed invention or, in the case of biological materials, by specifically describing a deposit made in accordance with 37 CFR 1.801 *et seq.* See also *Deposit of Biological Materials for Patent Purposes, Final Rule*, 54 FR 34,864 (August 22, 1989) ("The requirement for a specific identification is consistent with the description requirement of the first paragraph of 35 U.S.C. 112, and to provide an antecedent basis for the biological material which either has been or will be deposited before the patent is granted." *Id.* at 34,876. "The description must be sufficient to permit verification that the deposited biological material is in fact that disclosed. Once the

patent issues, the description must be sufficient to aid in the resolution of questions of infringement." *Id.* at 34,880. Such a deposit is not a substitute for a written description of the claimed invention. The written description of the deposited material needs to be as complete as possible because the examination for patentability proceeds solely on the basis of the written description. See, e.g., *In re Lundak*, 773 F.2d 1216, 227 USPQ 90 (Fed. Cir. 1985). See also 54 FR at 34,880 ("As a general rule, the more information that is provided about a particular deposited biological material, the better the examiner will be able to compare the identity and characteristics of the deposited biological material with the prior art.").

<sup>7</sup> *Pfaff v. Wells Electronics, Inc.*, 525 U.S. 55, 68, 119 S.Ct. 304, 312, 48 USPQ2d 1641, 1647 (1998); *Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406.

<sup>8</sup> See *Amgen, Inc. v. Chugai Pharmaceutical*, 927 F.2d 1200, 1206, 18 USPQ2d 1016, 1021 (Fed. Cir. 1991) (one must define a compound by "whatever characteristics sufficiently distinguish it").

<sup>9</sup> A description requirement issue can arise for original claims (see, e.g., *Eli Lilly*, 119 F.3d 1559, 43 USPQ2d 1398) as well as new or amended claims. Most typically, the issue will arise in the context of determining whether new or amended claims are supported by the description of the invention in the application as filed (see, e.g., *In re Wright*, 866 F.2d 422, 9 USPQ2d 1649 (Fed. Cir. 1989)), whether a claimed invention is entitled to the benefit of an earlier priority date or effective filing date under 35 U.S.C. 119, 120, or 365(c) (see, e.g., *Tronzo v. Biomet, Inc.*, 156 F.3d 1154, 47 USPQ2d 1829 (Fed. Cir. 1998); *Fiers v. Revel*, 984 F.2d 1164, 25 USPQ2d 1601 (Fed. Cir. 1993); *In re Ziegler*, 992 F.2d 1197, 1200, 26 USPQ2d 1600, 1603 (Fed. Cir. 1993)), or whether a specification provides support for a claim corresponding to a count in an interference (see, e.g., *Fields v. Conover*, 443 F.2d 1386, 170 USPQ 276 (CCPA 1971)).

<sup>10</sup> *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d at 1563, 19 USPQ2d at 1116 (Fed. Cir. 1991).

<sup>11</sup> *In re Wertheim*, 541 F.2d 257, 263, 191 USPQ 90, 97 (CCPA 1976) ("we are of the opinion that the PTO has the initial burden of presenting evidence or reasons why persons skilled in the art would not recognize in the disclosure a description of the invention defined by the claims").

<sup>12</sup> See endnote 4.

<sup>13</sup> For example, consider the claim "A gene comprising SEQ ID NO:1." A determination of what the claim as a whole covers may result in a conclusion that specific structures such as a promoter, a coding region, or other elements are included. Although all genes encompassed by this claim share the characteristic of comprising SEQ ID NO:1, there may be insufficient description of those specific structures (e.g., promoters, enhancers, coding regions, and other regulatory elements) which are also included.

<sup>14</sup> A biomolecule sequence described only by a functional characteristic, without any known or disclosed correlation between that function and the structure of the sequence, normally is not a sufficient identifying

characteristic for written description purposes, even when accompanied by a method of obtaining the claimed sequence. For example, even though a genetic code table would correlate a known amino acid sequence with a genus of coding nucleic acids, the same table cannot predict the native, naturally occurring nucleic acid sequence of a naturally occurring mRNA or its corresponding cDNA. Cf. *In re Bell*, 991 F.2d 781, 26 USPQ2d 1529 (Fed. Cir. 1993), and *In re Deuel*, 51 F.3d 1552, 34 USPQ2d 1210 (Fed. Cir. 1995) (holding that a process could not render the product of that process obvious under 35 U.S.C. 103). The Federal Circuit has pointed out that under United States law, a description that does not render a claimed invention obvious cannot sufficiently describe the invention for the purposes of the written description requirement of 35 U.S.C. 112. *Eli Lilly*, 119 F.3d at 1567, 43 USPQ2d at 1405.

*Compare Fonar Corp. v. General Electric Co.*, 107 F.3d 1543, 1549, 41 USPQ2d 1801, 1805 (Fed. Cir. 1997) ("As a general rule, where software constitutes part of a best mode of carrying out an invention, description of such a best mode is satisfied by a disclosure of the functions of the software. This is because, normally, writing code for such software is within the skill of the art, not requiring undue experimentation, once its functions have been disclosed. \* \* \* Thus, flow charts or source code listings are not a requirement for adequately disclosing the functions of software.").

<sup>15</sup> See, e.g., *Fujikawa v. Wattanasin*, 93 F.3d 1559, 1571, 39 USPQ2d 1895, 1905 (Fed. Cir. 1996) (a "laundry list" disclosure of every possible moiety does not constitute a written description of every species in a genus because it would not "reasonably lead" those skilled in the art to any particular species); *In re Ruschig*, 379 F.2d 990, 995, 154 USPQ 118, 123 (CCPA 1967) ("If n-propylamine had been used in making the compound instead of n-butylamine, the compound of claim 13 would have resulted. Appellants submit to us, as they did to the board, an imaginary specific example patterned on specific example 6 by which the above butyl compound is made so that we can see what a simple change would have resulted in a specific supporting disclosure being present in the present specification. The trouble is that there is no such disclosure, easy though it is to imagine it.") (emphasis in original); *Purdue Pharma L.P. v. Fausling Inc.*, 230 F.3d 1320, 1328, 56 USPQ2d 1481, 1487 (Fed. Cir. 2000) ("the specification does not clearly disclose to the skilled artisan that the inventors \* \* \* considered the [I] ratio to be part of their invention \* \* \*. There is therefore no force to Purdue's argument that the written description requirement was satisfied because the disclosure revealed a broad invention from which the [later-filed] claims carved out a patentable portion").

<sup>16</sup> 35 U.S.C. §§ 132 and 251. See also *In re Rasmussen*, 650 F.2d 1212, 1214, 211 USPQ 323, 326 (CCPA 1981). See Manual of Patent Examining Procedure (MPEP) §§ 2163.06-2163.07 (7th Ed., Rev. 1, Feb. 2000) for a more detailed discussion of the written description requirement and its relationship to new matter.

<sup>17</sup> The claims as filed in the original specification are part of the disclosure and, therefore, if an application as originally filed contains a claim disclosing material not found in the remainder of the specification, the applicant may amend the specification to include the claimed subject matter. *In re Benno*, 768 F.2d 1340, 226 USPQ 683 (Fed. Cir. 1985).

<sup>18</sup> See, e.g., *In re Lukach*, 442 F.2d 967, 169 USPQ 795 (CCPA 1971) (subgenus range was not supported by generic disclosure and specific example within the subgenus range); *In re Smith*, 458 F.2d 1389, 1395, 173 USPQ 679, 683 (CCPA 1972) (a subgenus is not necessarily described by a genus encompassing it and a species upon which it reads).

<sup>19</sup> *In re Oda*, 443 F.2d 1200, 170 USPQ 260 (CCPA 1971). With respect to the correction of sequencing errors in applications disclosing nucleic acid and/or amino acid sequences, it is well known that sequencing errors are a common problem in molecular biology. See, e.g., Peter Richterich, *Estimation of Errors in 'Raw' DNA Sequences: A Validation Study*, 8 Genome Research 251-59 (1998). If an application as filed includes sequence information and references a deposit of the sequenced material made in accordance with the requirements of 37 CFR § 1.801 *et seq.*, amendment may be permissible.

<sup>20</sup> Corrections of minor errors in the sequence may be possible based on the argument that one of skill in the art would have resequenced the deposited material and would have immediately recognized the minor error. Deposits made after the filing date can only be relied upon to provide support for the correction of sequence information if applicant submits a statement in compliance with 37 CFR § 1.804 stating that the biological material which is deposited is a biological material specifically defined in the application as filed.

<sup>21</sup> See, e.g., *Gentry Gallery, Inc. v. Berkline Corp.*, 134 F.3d 1473, 45 USPQ2d 1498 (Fed. Cir. 1998) (claims to a sectional sofa comprising, *inter alia*, a console and a control means were held invalid for failing to satisfy the written description requirement where the claims were broadened by removing the location of the control means.); *Johnson Worldwide Associates v. Zebco Corp.*, 175 F.3d 985, 993, 50 USPQ2d 1607, 1613 (Fed. Cir. 1999) (In *Gentry Gallery*, the "court's determination that the patent disclosure did not support a broad meaning for the disputed claim terms was premised on clear statements in the written description that described the location of a claim element—the 'control means'—as 'the only possible location' and that variations were 'outside the stated purpose of the invention.' *Gentry Gallery*, 134 F.3d at 1479, 45 USPQ2d at 1503. *Gentry Gallery*, then, considers the situation where the patent's disclosure makes crystal clear that a particular (*i.e.*, narrow) understanding of a claim term is an 'essential element of [the inventor's] invention.'"); *Tronzo v. Biomet*, 156 F.3d at 1158-59, 47 USPQ2d at 1833 (Fed. Cir. 1998) (claims to generic cup shape were not entitled to filing date of parent application which disclosed "conical cup" in view of the disclosure of the



parent application stating the advantages and importance of the conical shape.)

<sup>22</sup> See *Gentry Gallery*, 134 F.3d at 1480, 45 USPQ2d at 1503; *In re Sus*, 306 F.2d 494, 504, 134 USPQ 301, 309 (CCPA 1962) ("[O]ne skilled in this art would not be taught by the written description of the invention in the specification that any 'aryl or substituted aryl radical' would be suitable for the purposes of the invention but rather that only *certain aryl radicals* and certain specifically substituted aryl radicals [i.e., aryl azides] would be suitable for such purposes.") (emphasis in original). A claim which omits matter disclosed to be essential to the invention as described in the specification or in other statements of record may also be subject to rejection under 35 U.S.C. 112, ¶ 1, as not enabling, or under 35 U.S.C. 112, ¶ 2. See *In re Mayhew*, 527 F.2d 1229, 188 USPQ 356 (CCPA 1976); *In re Venezia*, 530 F.2d 956, 189 USPQ 149 (CCPA 1976); and *In re Collier*, 397 F.2d 1003, 158 USPQ 266 (CCPA 1968). See also MPEP § 2172.01.

<sup>23</sup> See, e.g., *Vas-Cath, Inc.*, 935 F.2d at 1563-64, 19 USPQ2d at 1117.

<sup>24</sup> *Wertheim*, 541 F.2d at 262, 191 USPQ at 96.

<sup>25</sup> See MPEP §§ 714.02 and 2163.06 ("Applicant should \* \* \* specifically point out the support for any amendments made to the disclosure."); and MPEP § 2163.04 ("If applicant amends the claims and points out where and/or how the originally filed disclosure supports the amendment(s), and the examiner finds that the disclosure does not reasonably convey that the inventor had possession of the subject matter of the amendment at the time of the filing of the application, the examiner has the initial burden of presenting evidence or reasoning to explain why persons skilled in the art would not recognize in the disclosure a description of the invention defined by the claims.").

<sup>26</sup> See *In re Smith*, 458 F.2d 1389, 1395, 173 USPQ 679, 683 (CCPA 1972) ("Precisely how close [to the claimed invention] the description must come to comply with § 112 must be left to case-by-case development."); *In re Wertheim*, 541 F.2d at 262, 191 USPQ at 96 (inquiry is primarily factual and depends on the nature of the invention and the amount of knowledge imparted to those skilled in the art by the disclosure).

<sup>27</sup> See, e.g., *In re Morris*, 127 F.3d 1048, 1053-54, 44 USPQ2d 1023, 1027 (Fed. Cir. 1997).

<sup>28</sup> "Preamble language" is that language in a claim appearing before the transitional phrase, e.g., before "comprising," "consisting essentially of," or "consisting of."

<sup>29</sup> The transitional term "comprising" (and other comparable terms, e.g., "containing," "including," and "having") is "open-ended—it covers the expressly recited subject matter, alone or in combination with unrecited subject matter. See, e.g., *Genentech, Inc. v. Chiron Corp.*, 112 F.3d 495, 501, 42 USPQ2d 1608, 1613 (Fed. Cir. 1997) ("Comprising" is a term of art used in claim language which means that the named elements are essential, but other elements may be added and still form a construct within the scope of the claim."); *Ex parte Davis*, 80 USPQ 448, 450 (Bd. App. 1948) ("comprising" leaves the

"claim open for the inclusion of unspecified ingredients even in major amounts"). "By using the term 'consisting essentially of,' the drafter signals that the invention necessarily includes the listed ingredients and is open to unlisted ingredients that do not materially affect the basic and novel properties of the invention. A 'consisting essentially of' claim occupies a middle ground between closed claims that are written in a 'consisting of' format and fully open claims that are drafted in a 'comprising' format." *PPG Industries v. Guardian Industries*, 156 F.3d 1351, 1354, 48 USPQ2d 1351, 1353-54 (Fed. Cir. 1998). For the purposes of searching for and applying prior art under 35 U.S.C. 102 and 103, absent a clear indication in the specification or claims of what the basic and novel characteristics actually are, 'consisting essentially of' will be construed as equivalent to "comprising." See, e.g., *PPG*, 156 F.3d at 1355, 48 USPQ2d at 1355 ("PPG could have defined the scope of the phrase 'consisting essentially of' for purposes of its patent by making clear in its specification what it regarded as constituting a material change in the basic and novel characteristics of the invention."). See also *In re Janakirama-Rao*, 317 F.2d 951, 954, 137 USPQ 893, 895-96 (CCPA 1963). If an applicant contends that additional steps or materials in the prior art are excluded by the recitation of "consisting essentially of," applicant has the burden of showing that the introduction of additional steps or components would materially change the characteristics of applicant's invention. *In re De Lajarte*, 337 F.2d 870, 143 USPQ 256 (CCPA 1964).

<sup>30</sup> See *Pac-Tec Inc. v. Amerace Corp.*, 903 F.2d 796, 801, 14 USPQ2d 1871, 1876 (Fed. Cir. 1990) (determining that preamble language that constitutes a structural limitation is actually part of the claimed invention).

<sup>31</sup> An applicant shows possession of the claimed invention by describing the claimed invention with all of its limitations. *Lockwood*, 107 F.3d at 1572, 41 USPQ2d at 1966.

<sup>32</sup> See, e.g., *Bell Communications Research, Inc. v. Vitalink Communications Corp.*, 55 F.3d 615, 620, 34 USPQ2d 1816, 1820 (Fed. Cir. 1995) ("[A] claim preamble has the import that the claim as a whole suggests for it."); *Corning Glass Works v. Sumitomo Elec. U.S.A., Inc.*, 868 F.2d 1251, 1257, 9 USPQ2d 1962, 1966 (Fed. Cir. 1989) (The determination of whether preamble recitations are structural limitations can be resolved only on review of the entirety of the application "to gain an understanding of what the inventors actually invented and intended to encompass by the claim.").

<sup>33</sup> An element may be critical where those of skill in the art would require it to determine that applicant was in possession of the invention. *Compare Rasmussen*, 650 F.2d at 1215, 211 USPQ at 327 ("one skilled in the art who read Rasmussen's specification would understand that it is unimportant how the layers are adhered, so long as they are adhered") (emphasis in original), with *Amgen, Inc. v. Chugai Pharmaceutical Co., Ltd.*, 927 F.2d 1200, 1206, 18 USPQ2d 1016, 1021 (Fed. Cir. 1991) ("it is well established in our law that conception of a chemical

compound requires that the inventor be able to define it so as to distinguish it from other materials, and to describe how to obtain it").

<sup>34</sup> See, e.g., *Wang Labs. v. Toshiba Corp.*, 993 F.2d 858, 865, 26 USPQ2d 1767, 1774 (Fed. Cir. 1993).

<sup>35</sup> See, e.g., *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1379-80, 231 USPQ 81, 90 (Fed. Cir. 1986).

<sup>36</sup> See, e.g., *Purdue Pharma L.P. v. Faulding Inc.*, 230 F.3d 1320, \_\_\_, 56 USPQ2d 1481, 1483 (Fed. Cir. 2000) (the written description "inquiry is a factual one and must be assessed on a case-by-case basis"); see also *Pfaff v. Wells Electronics, Inc.*, 55 U.S. at 66, 119 S.Ct. at 311, 48 USPQ2d at 1646 ("The word 'invention' must refer to a concept that is complete, rather than merely one that is 'substantially complete.' It is true that reduction to practice ordinarily provides the best evidence that an invention is complete. But just because reduction to practice is sufficient evidence of completion, it does not follow that proof of reduction to practice is necessary in every case. Indeed, both the facts of the *Telephone Cases* and the facts of this case demonstrate that one can prove that an invention is complete and ready for patenting before it has actually been reduced to practice.").

<sup>37</sup> *Cooper v. Goldfarb*, 154 F.3d 1321, 1327, 47 USPQ2d 1896, 1901 (Fed. Cir. 1998). See also *UMC Elecs. Co. v. United States*, 816 F.2d 647, 652, 2 USPQ2d 1465, 1468 (Fed. Cir. 1987) ("[T]here cannot be a reduction to practice of the invention \* \* \* without a physical embodiment which includes all limitations of the claim."); *Estee Lauder Inc. v. L'Oreal, S.A.*, 129 F.3d 588, 593, 44 USPQ2d 1610, 1614 (Fed. Cir. 1997) ("[A] reduction to practice does not occur until the inventor has determined that the invention will work for its intended purpose."); *Mahurkar v. C.R. Bard, Inc.*, 79 F.3d 1572, 1578, 38 USPQ2d 1288, 1291 (Fed. Cir. 1996) (determining that the invention will work for its intended purpose may require testing depending on the character of the invention and the problem it solves).

<sup>38</sup> 37 CFR 1.804, 1.809. See also endnote 6.

<sup>39</sup> See, e.g., *Vas-Cath*, 935 F.2d at 1565, 19 USPQ2d at 1118 ("drawings alone may provide a 'written description' of an invention as required by § 112"); *In re Wolfensperger*, 302 F.2d 950, 133 USPQ 537 (CCPA 1962) (the drawings of applicant's specification provided sufficient written descriptive support for the claim limitation at issue); *Autogiro Co. of America v. United States*, 384 F.2d 391, 398, 155 USPQ 697, 703 (Ct. Cl. 1967) ("In those instances where a visual representation can flesh out words, drawings may be used in the same manner and with the same limitations as the specification.").

<sup>40</sup> See, e.g., *Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406 ("In claims involving chemical materials, generic formulae usually indicate with specificity what the generic claims encompass. One skilled in the art can distinguish such a formula from others and can identify many of the species that the claims encompass. Accordingly, such a formula is normally an adequate description of the claimed genus.").

<sup>41</sup> See *Hybritech v. Monoclonal Antibodies*, 802 F.2d at 1384, 231 USPQ at 94; *Fonar Corp. v. General Electric Co.*, 107 F.3d at 1549, 41 USPQ2d at 1805 (source code description not required).

<sup>42</sup> For example, the presence of a restriction enzyme map of a gene may be relevant to a statement that the gene has been isolated. One skilled in the art may be able to determine when the gene disclosed is the same as or different from a gene isolated by another by comparing the restriction enzyme map. In contrast, evidence that the gene could be digested with a nuclease would not normally represent a relevant characteristic since any gene would be digested with a nuclease. Similarly, isolation of an mRNA and its expression to produce the protein of interest is strong evidence of possession of an mRNA for the protein.

For some biomolecules, examples of identifying characteristics include a sequence, structure, binding affinity, binding specificity, molecular weight, and length. Although structural formulas provide a convenient method of demonstrating possession of specific molecules, other identifying characteristics or combinations of characteristics may demonstrate the requisite possession. For example, unique cleavage by particular enzymes, isoelectric points of fragments, detailed restriction enzyme maps, a comparison of enzymatic activities, or antibody cross-reactivity may be sufficient to show possession of the claimed invention to one of skill in the art. See *Lockwood*, 107 F.3d at 1572, 41 USPQ2d at 1966 ("written description" requirement may be satisfied by using "such descriptive means as words, structures, figures, diagrams, formulas, etc., that fully set forth the claimed invention").

<sup>43</sup> A definition by function alone "does not suffice" to sufficiently describe a coding sequence "because it is only an indication of what the gene does, rather than what it is." *Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406. See also *Fiers*, 984 F.2d at 1169-71, 25 USPQ2d at 1605-06 (discussing *Amgen Inc. v. Chugai Pharmaceutical Co.*, 927 F.2d 1200, 18 USPQ2d 1016 (Fed. Cir. 1991)).

<sup>44</sup> If a claim limitation invokes 35 U.S.C. 112, ¶ 6, it must be interpreted to cover the corresponding structure, materials, or acts in the specification and "equivalents thereof." See 35 U.S.C. 112, ¶ 6. See also *B. Braun Medical, Inc. v. Abbott Lab.*, 124 F.3d 1419, 1424, 43 USPQ2d 1896, 1899 (Fed. Cir. 1997). In considering whether there is 35 U.S.C. 112, ¶ 1, support for a means- (or step-) plus-function claim limitation, the examiner must consider not only the original disclosure contained in the summary and detailed description of the invention portions of the specification, but also the original claims, abstract, and drawings. A means- (or step-) plus-function claim limitation is adequately described under 35 U.S.C. 112, ¶ 1, if: (1) The written description adequately links or associates adequately described particular structure, material, or acts to the function recited in a means- (or step-) plus-function claim limitation; or (2) it is clear based on the facts of the application that one skilled in the art would have known what structure, material, or acts perform the function recited in a means- (or step-) plus-

function limitation. Note also: A rejection under 35 U.S.C. 112, ¶ 2, "cannot stand where there is adequate description in the specification to satisfy 35 U.S.C. 112, first paragraph, regarding means-plus-function recitations that are not, per se, challenged for being unclear." *In re Noll*, 545 F.2d 141, 149, 191 USPQ 721, 727 (CCPA 1976). See *Supplemental Examination Guidelines for Determining the Applicability of 35 U.S.C. 112, ¶ 6*, 65 FR 38510, June 21, 2000.

<sup>45</sup> See *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d at 1384, 231 USPQ at 94.

<sup>46</sup> See, e.g., *Vas-Cath*, 935 F.2d at 1563, 19 USPQ2d at 1116; *Martin v. Johnson*, 454 F.2d 746, 751, 172 USPQ 391, 395 (CCPA 1972) (stating "the description need not be in *ipsis verbis* [i.e., "in the same words"] to be sufficient").

<sup>47</sup> A claim which is limited to a single disclosed embodiment or species is analyzed as a claim drawn to a single embodiment or species, whereas a claim which encompasses two or more embodiments or species within the scope of the claim is analyzed as a claim drawn to a genus. See also MPEP § 806.04(e).

<sup>48</sup> 35 U.S.C. 112, ¶ 1. *Cf. Fields v. Conover*, 443 F.2d 1386, 1392, 170 USPQ 276, 280 (CCPA 1971) (finding a lack of written description because the specification lacked the "full, clear, concise, and exact written description" which is necessary to support the claimed invention).

<sup>49</sup> For example, if the art has established a strong correlation between structure and function, one skilled in the art would be able to predict with a reasonable degree of confidence the structure of the claimed invention from a recitation of its function. Thus, the written description requirement may be satisfied through disclosure of function and minimal structure when there is a well-established correlation between structure and function. In contrast, without such a correlation, the capability to recognize or understand the structure from the mere recitation of function and minimal structure is highly unlikely. In this latter case, disclosure of function alone is little more than a wish for possession; it does not satisfy the written description requirement. See *Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406 (written description requirement not satisfied by merely providing "a result that one might achieve if one made that invention"); *In re Wilder*, 736 F.2d 1516, 1521, 222 USPQ 369, 372-73 (Fed. Cir. 1984) (affirming a rejection for lack of written description because the specification does "little more than outline goals appellants hope the claimed invention achieves and the problems the invention will hopefully ameliorate"). Compare *Fonar*, 107 F.3d at 1549, 41 USPQ2d at 1805 (disclosure of software function adequate in that art).

<sup>50</sup> See *Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406.

<sup>51</sup> See, e.g., *In re Hayes Microcomputer Products, Inc. Patent Litigation*, 982 F.2d 1527, 1534-35, 25 USPQ2d 1241, 1246 (Fed. Cir. 1992) ("One skilled in the art would know how to program a microprocessor to perform the necessary steps described in the specification. Thus, an inventor is not required to describe every detail of his invention. An applicant's disclosure

obligation varies according to the art to which the invention pertains. Disclosing a microprocessor capable of performing certain functions is sufficient to satisfy the requirement of section 112, first paragraph, when one skilled in the relevant art would understand what is intended and know how to carry it out.")

<sup>52</sup> See, e.g., *Fiers v. Revel*, 984 F.2d at 1169, 25 USPQ2d at 1605; *Amgen*, 927 F.2d at 1206, 18 USPQ2d at 1021. Where the process has actually been used to produce the product, the written description requirement for a product-by-process claim is clearly satisfied; however, the requirement may not be satisfied where it is not clear that the acts set forth in the specification can be performed, or that the product is produced by that process.

<sup>53</sup> See, e.g., *Amgen*, 927 F.2d at 1206, 18 USPQ2d at 1021 ("A gene is a chemical compound, albeit a complex one, and it is well established in our law that conception of a chemical compound requires that the inventor be able to define it so as to distinguish it from other materials, and to describe how to obtain it. Conception does not occur unless one has a mental picture of the structure of the chemical, or is able to define it by its method of preparation, its physical or chemical properties, or whatever characteristics sufficiently distinguish it. It is not sufficient to define it solely by its principal biological property, e.g., encoding human erythropoietin, because an alleged conception having no more specificity than that is simply a wish to know the identity of any material with that biological property. We hold that when an inventor is unable to envision the detailed constitution of a gene so as to distinguish it from other materials, as well as a method for obtaining it, conception has not been achieved until reduction to practice has occurred, i.e., until after the gene has been isolated.") (citations omitted). In such instances the alleged conception fails not merely because the field is unpredictable or because of the general uncertainty surrounding experimental sciences, but because the conception is incomplete due to factual uncertainty that undermines the specificity of the inventor's idea of the invention. *Burroughs Wellcome Co. v. Barr Laboratories Inc.*, 40 F.3d 1223, 1229, 32 USPQ2d 1915, 1920 (Fed. Cir. 1994). Reduction to practice in effect provides the only evidence to corroborate conception (and therefore possession) of the invention. *Id.*

<sup>54</sup> See *Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406.

<sup>55</sup> See, e.g., *Rasmussen*, 650 F.2d at 1214, 211 USPQ at 326-27 (disclosure of a single method of adhering applying one layer to another was sufficient to support a generic claim to "adhering applying" because one skilled in the art reading the specification would understand that it is unimportant how the layers are adhered, so long as they are adhered); *In re Herschler*, 591 F.2d 693, 697, 200 USPQ 711, 714 (CCPA 1979) (disclosure of corticosteroid in DMSO sufficient to support claims drawn to a method of using a mixture of a "physiologically active steroid" and DMSO because "use of known chemical compounds in a manner auxiliary

to the invention must have a corresponding written description only so specific as to lead one having ordinary skill in the art to that class of compounds. Occasionally, a functional recitation of those known compounds in the specification may be sufficient as that description."'); *In re Smythe*, 480 F.2d 1376, 1383, 178 USPQ 279, 285 (CCPA 1973) (the phrase "air or other gas which is inert to the liquid" was sufficient to support a claim to "inert fluid media" because the description of the properties and functions of the air or other gas segmentizing medium would suggest to a person skilled in the art that appellant's invention includes the use of "inert fluid" broadly.). However, in *Tronzo v. Biomet*, 156 F.3d at 1159, 47 USPQ2d at 1833 (Fed. Cir. 1998), the disclosure of a species in the parent application did not suffice to provide written description support for the genus in the child application.

<sup>56</sup> See, e.g., *Eli Lilly*.

<sup>57</sup> For example, in the molecular biology arts, if an applicant disclosed an amino acid sequence, it would be unnecessary to provide an explicit disclosure of nucleic acid sequences that encoded the amino acid sequence. Since the genetic code is widely known, a disclosure of an amino acid sequence would provide sufficient information such that one would accept that an applicant was in possession of the full genus of nucleic acids encoding a given amino acid sequence, but not necessarily any particular species. Cf. *In re Bell*, 991 F.2d 781, 785, 26 USPQ2d 1529, 1532 (Fed. Cir. 1993) and *In re Baird*, 16 F.3d 380, 382, 29 USPQ2d 1550, 1552 (Fed. Cir. 1994).

<sup>58</sup> See *Wertheim*, 541 F.2d at 263, 191 USPQ at 97 ("[T]he PTO has the initial burden of presenting evidence or reasons why persons skilled in the art would not recognize in the disclosure a description of the invention defined by the claims.").

<sup>59</sup> See MPEP §§ 714.02 and 2163.06 ("Applicant should \* \* \* specifically point out the support for any amendments made to the disclosure.").

<sup>60</sup> See, e.g., *In re Wright*, 866 F.2d 422, 425, 9 USPQ2d 1649, 1651 (Fed. Cir. 1989) (Original specification for method of forming images using photosensitive microcapsules which describes removal of microcapsules from surface and warns that capsules not be disturbed prior to formation of image, unequivocally teaches absence of permanently fixed microcapsules and supports amended language of claims requiring that microcapsules be "not permanently fixed" to underlying surface, and therefore meets description requirement of 35 U.S.C. 112.).

<sup>61</sup> See, e.g., *In re Robins*, 429 F.2d 452, 456-57, 166 USPQ 552, 555 (CCPA 1970) ("[W]here no explicit description of a generic invention is to be found in the specification \* \* \* mention of representative compounds may provide an implicit description upon which to base generic claim language."); *In re Smith*, 458 F.2d 1389, 1395, 173 USPQ 679, 683 (CCPA 1972) (a subgenus is not necessarily implicitly described by a genus encompassing it and a species upon which it reads).

<sup>62</sup> See, e.g., *In re Robertson*, 169 F.3d 743, 745, 49 USPQ2d 1949, 1950-51 (Fed. Cir.

1999) ("To establish inherency, the extrinsic evidence "must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill. Inherency, however, may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient."") (citations omitted).

<sup>63</sup> When an explicit limitation in a claim "is not present in the written description whose benefit is sought it must be shown that a person of ordinary skill would have understood, at the time the patent application was filed, that the description requires that limitation." *Hyatt v. Boone*, 146 F.3d 1348, 1353, 47 USPQ2d 1128, 1131 (Fed. Cir. 1998).

<sup>64</sup> See, e.g., *Johnson Worldwide Associates Inc. v. Zebco Corp.*, 175 F.3d at 993, 50 USPQ2d at 1613; *Gentry Gallery, Inc. v. Berkline Corp.*, 134 F.3d at 1479, 45 USPQ2d at 1503; *Tronzo v. Biomet*, 156 F.3d at 1159, 47 USPQ2d at 1833.

<sup>65</sup> See, e.g., *In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971).

<sup>66</sup> *Wertheim*, 541 F.2d at 263, 191 USPQ at 97.

<sup>67</sup> See *Rasmussen*, 650 F.2d at 1214, 211 USPQ at 326.

<sup>68</sup> See *In re Alton*, 76 F.3d 1168, 1176, 37 USPQ2d 1578, 1584 (Fed. Cir. 1996).

[FR Doc. 01-323 Filed 1-4-01; 8:45 am]

BILLING CODE 3510-16-U

## CORPORATION FOR NATIONAL AND COMMUNITY SERVICE

### Revision of Currently Approved Information Collection; Comment Request

AGENCY: Corporation for National and Community Service

ACTION: Notice.

**SUMMARY:** The Corporation for National and Community Service (hereinafter "Corporation"), as part of its continuing effort to reduce paperwork and respondent burden, conducts a preclearance consultation program to provide the general public and Federal agencies with an opportunity to comment on proposed and/or continuing collections of information in accordance with the Paperwork Reduction Act of 1995 (PRA95) (44 U.S.C. 3506(c)(2)(A)). This program helps to ensure that requested data can be provided in the desired format, reporting burden (time and financial resources) is minimized, collection instruments are clearly understood, and the impact of collection requirement on respondents can be properly assessed.

Currently, the Corporation is soliciting comments concerning the proposed revision of its Voucher and

Payment Request Form (OMB #3045-0014).

Copies of the forms can be obtained by contacting the office listed below in the address section of this notice.

**DATES:** Written comments must be submitted to the office listed in the ADDRESSES section by March 6, 2001.

**ADDRESSES:** Send comments to Levon Buller, National Service Trust, Corporation for National and Community Service, 1201 New York Ave., NW., Washington, DC 20525.

**FOR FURTHER INFORMATION CONTACT:** Levon Buller, (202) 606-5000, ext. 383.

**SUPPLEMENTARY INFORMATION:** The Corporation is particularly interested in comments which:

- Evaluate whether the proposed collection of information is necessary for the proper performance of the functions of the Corporation, including whether the information will have practical utility;
- Evaluate the accuracy of the agency's estimate of the burden of the proposed collection of information, including the validity of the methodology and assumptions used;
- Enhance the quality, utility and clarity of the information to be collected; and
- Minimize the burden of the collection of information on those who are to respond, including through the use of appropriate automated, electronic, mechanical, or other technological collection techniques or other forms of information technology, e.g., permitting electronic submissions of responses.

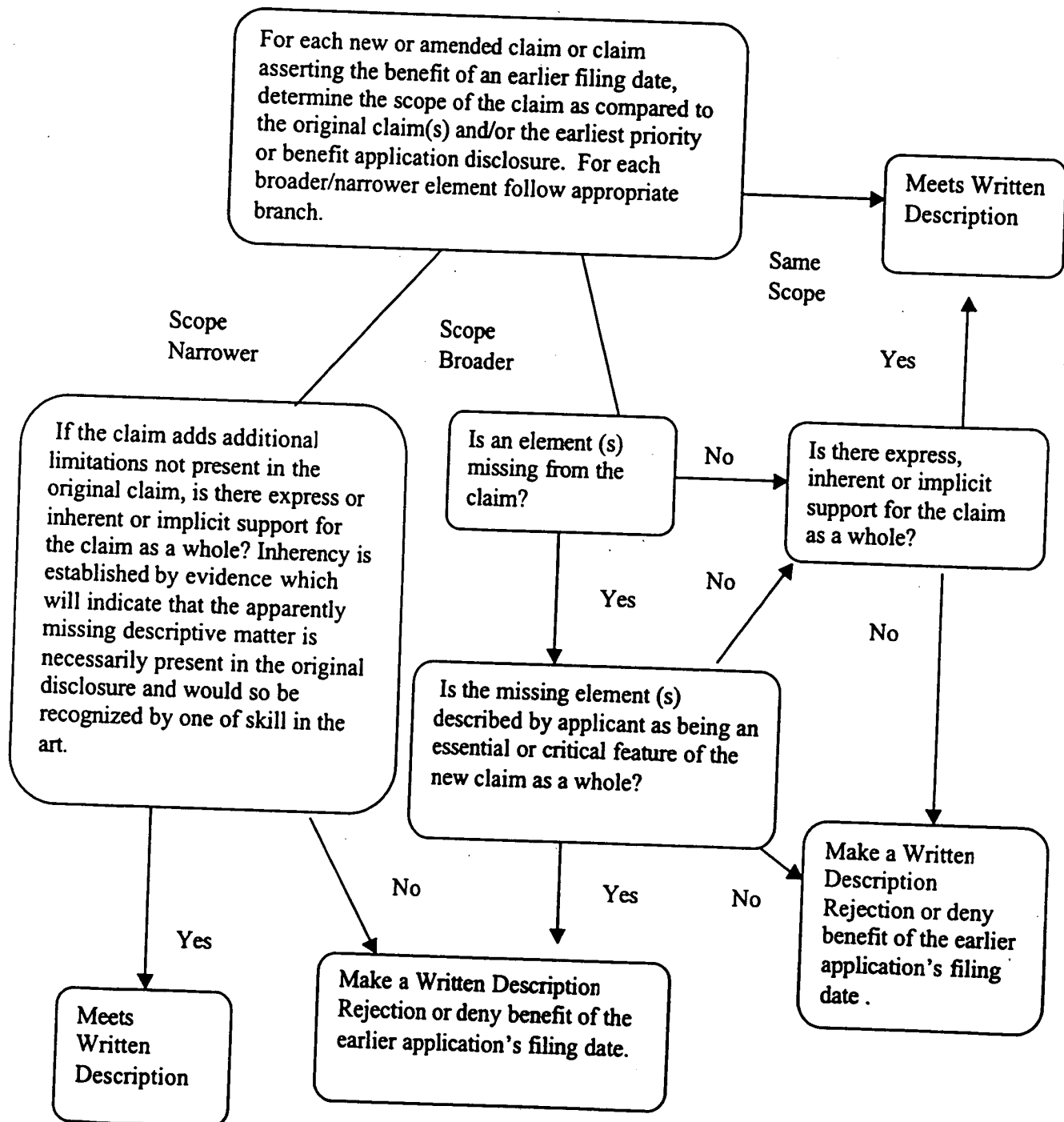
### Background

The Corporation supports programs that provide opportunities for individuals who want to become involved in national service. The service opportunities cover a wide range of activities over varying periods of time. Upon successfully completing an agreed-upon term of service in an approved AmeriCorps program, a national service participant—an AmeriCorps member—receives an "education award". This award is an amount of money set aside in the member's name in the National Service Trust Fund. This education award can be used to make payments towards qualified student loan or pay for educational expenses at qualified post-secondary institutions and approved school-to-work opportunities programs. Members have seven years in which to draw against any unused balance.

The National Service Trust is the office within the Corporation that administers the education award

**Written Description Amended**  
**or New Claims, or Claims Asserting**  
**the Benefit of an Earlier Filing Date**

**Decision Tree**



Arylsulfonyltetrazoles, new coupling reagents and further improvements in the triester method for the synthesis of deoxyribooligonucleotides<sup>1</sup>

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<sup>a</sup>Division of Biological Sciences<sup>2</sup>, National Research Council of Canada, Ottawa, Canada K1A 0R6 and <sup>b</sup>Section of Biochemistry, Molecular and Cell Biology<sup>2</sup>, Cornell Univ., Ithaca, NY 14853, USA

Received 16 November 1976

### ABSTRACT

The modified triester approach has been further improved and refined to the synthesis of defined sequences of deoxyribooligonucleotides. Improvements include arylsulfonyltetrazoles as faster and milder condensing agents, benzenesulfonic acid to avoid depurination during deblocking of trityl protecting groups and improved chromatographic procedures for purification of triester intermediates and purification of the final product containing 3'-5' phosphodiester linkages.

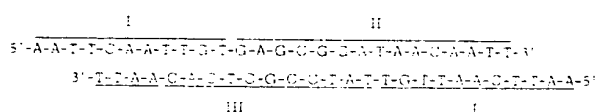
The efficiency of these new modifications in terms of yields and time has been illustrated by the synthesis of various defined sequences related to the lactose-operator region of *E. coli*, its analogues and two restriction enzyme recognition regions.

### INTRODUCTION

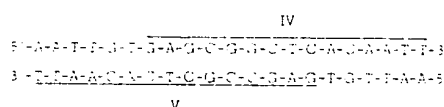
The synthetic availability of various deoxyribooligonucleotides containing the sequence of various control regions of a gene could be of great importance in the study of the mechanism of sequence specific DNA-protein interaction. The triester chemical method<sup>3</sup> seems to offer the first opportunity for large scale synthesis of oligonucleotides. Previously, we reported on the use of modified triester method developed in our laboratory for the chemical synthesis of a 21-mer duplex with demonstrated biological activity of the lactose operator gene of *E. coli*<sup>4,1</sup>. During these studies we developed arylsulfonyltetrazoles<sup>5</sup> as new coupling reagents which were found to give much higher yields of condensation products especially in the synthesis of oligonucleotides containing guanine base. However, these reagents were rather slow in completing the condensation reactions (3-5 days)<sup>1</sup>. In this paper we wish to report on:

(i) arylsulfonyltetrazoles as new and more reactive coupling reagents; (ii) further improvements in the triester approach to remove unreacted starting material; and (iii) purification of the final diester products by PEI-TLC plate<sup>6</sup>. The usefulness of these modifications has been demonstrated by the efficient synthesis of various deoxyribooligonucleotides constituting the duplex sequence of the lac-operator region<sup>7</sup> containing Eco RI restriction endonuclease recognition sequence<sup>8</sup> (A), symmetrical lac-operator (B), its bromouracil analogues<sup>9</sup> (C), decanucleotide containing the Bam I restriction enzyme sequence<sup>8,10</sup> (D), and second decanucleotide containing the Hind III restriction enzyme recognition sequence<sup>8,11</sup> (E) as outlined in Figure 1. The sequence of these oligonucleotides was confirmed by electrophoresis homochromatography mobility-shift analysis<sup>12</sup>.

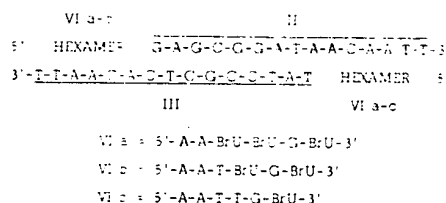
A. LACTOSE-OPERATOR CONTAINING Eco RI RECOGNITION SEQUENCE



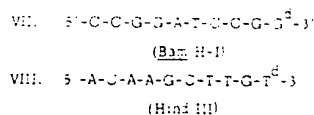
B. SYMMETRICAL LACTOSE OPERATOR



C. LACTOSE-OPERATOR BRU-URACIL ANALOGUES



D. RESTRICTION ENZYME RECOGNITION SEQUENCES

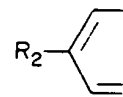


<sup>d</sup>These sequences are self-complementary.

Figure 1. The nucleotide sequence of the lac operator and restriction enzyme recognition sites.

RESULTS  
Arylsulfo

To  
mesityl  
80% yield  
tetrazole  
2 hr. B  
yield af  
benzene  
vacuo to  
the benz  
within 1  
of conden  
was depe  
phenyl  
and all  
triisopr  
Further  
F  
approach  
material  
in order  
condensa  
5'-termi  
protecti  
base lac  
conditio



- a. BS-T
- b. MS-T
- c. TPS-

Figure 1

**RESULTS AND DISCUSSION****Arylsulfonyltetrazoles Coupling Reagents**

Triisopropylbenzenesulfonyltetrazole (TPS-tetr) and mesitylenesulfonyltetrazole (MS-tetr) were directly prepared in 80% yield by treating the respective arylsulfonyl chloride with tetrazole in dioxane solution containing triethylamine at 5° for 2 hr. Benzenesulfonyltetrazole (BS-tetr) was obtained in 60-70% yield after purification in crystalline form by shaking its benzene solution with silica-gel, filtration and evaporation in vacuo to dryness. All these compounds (Figure 2) and especially the benzenesulfonyltetrazole were found to decompose on storage within 20 days. Comparison of the time periods for the completion of condensation reaction indicated that the rate of condensation was dependent upon the substitution in the benzene ring<sup>13</sup>, i.e. phenyl > 2,4,6-trimethylphenyl > 2,4,6-trisopropylphenyl groups and all these reagents were more reactive than triazoles and even triisopropyl benzenesulfonyl chloride<sup>14</sup>.

**Further Modification in the Triester Approach**

Previously, we have modified the triester synthetic approach<sup>4</sup> by introducing two key steps, i.e. (i) the starting material was a fully protected mononucleotide-3'-phosphotriester in order to eliminate the phosphorylation step before each condensation, and (ii) the chain was grown from the 3'-end towards 5'-terminus thus requiring the deblocking of an acid labile protecting group (monomethoxy- or dimethoxytrityl) instead of a base labile  $\beta$ -cyanoethyl group. Increasingly stronger basic conditions were required to deblock the  $\beta$ -cyanoethyl group in

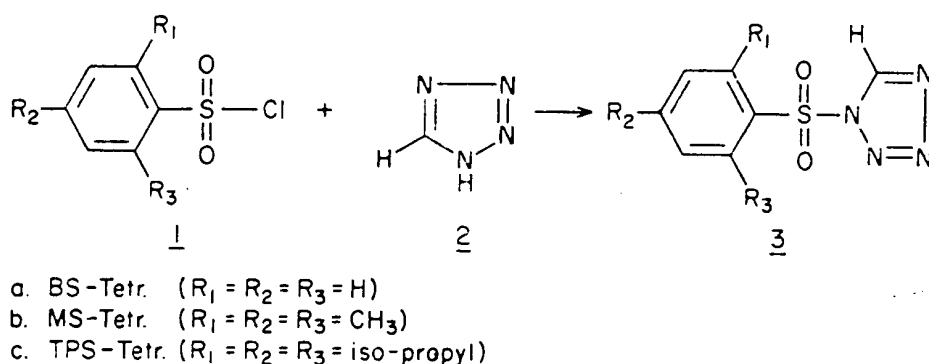


Figure 2. Synthesis of various arylsulfonyltetrazoles.

longer chain oligonucleotides. Using the modified triester approach, we occasionally observed that the pure fully protected product could not be separated quantitatively from the unreacted starting component containing 5'-primary hydroxyl groups, especially in the case of longer chains containing several guanine bases. Phosphorylation of the 5'-hydroxyl component followed by rechromatography on silica-gel should cause the oligonucleotide component containing the negatively charged phosphate group to be retained more strongly on silica-gel. We first tried phosphorus-oxychloride for phosphorylation - but obtained a neutral product, probably a phosphodichloridate. Attempted phosphorylation with  $\delta$ -cyanoethyl phosphate in the presence of benzenesulfonyltetrazole yielded again a triester<sup>15</sup> but in the presence of benzenesulfonyltriazole, the desired phosphodiester group was introduced quantitatively.

#### Benzenesulfonic Acid, A New Detritylating Reagent

The removal of trityl protecting groups from fully protected deoxyriboologonucleotides with 80% acetic acid causes extensive depurination and the production of numerous side products during synthesis by the modified triester approach. In order to overcome this problem we searched for a new deblocking reagent which would be able to hydrolyze the ether bond of trityl protecting groups much faster than the glycosidic bond in fully protected deoxyriboologonucleotides containing purine bases. We reasoned that an aromatic acid would have an affinity for the aromatic rings of the trityl group this localizing the acid catalysis to this part of the molecule. Indeed, we found that (Table I) benzenesulfonic acid as 2% solution in chloroform selectively removed monomethoxy or dimethoxytrityl groups at 0°C in 20 min and 3 min respectively with a minimum of depurination. Under the above conditions, all the other protecting groups were found to be stable. Comparative data with classical reagent for detritylation, 80% acetic acid is given in Table I.

#### Synthesis of Deoxyriboologonucleotides

The general plan for the synthesis is outlined in Figure 3. The 3'-hydroxyl of a starting nucleoside was protected with the benzoyl instead of the acetyl group because of the high solubility of the fully protected compounds in chloroform, faster mobility

Table

#### Compounds

##### Reagent

2% BSA in  
CHCl<sub>3</sub>/MeOH  
7:3

1% BSA in  
CHCl<sub>3</sub>/MeOH  
7:3

80% AcOH

\* Yield was 45

† Depurinated

‡ Expected det.

a After 20 min

b After 3 min

on silica-  
its struct  
3'-O-benzy  
dimethoxyt  
(p-chlorop  
in the pre  
based on 5  
The reacti  
followed b  
triethylam  
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eluent. T  
TLC were p  
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Table 1. Removal of Mono- and Dimethoxytrityl Group with Benzenesulfonic Acid (BSA) and Its Comparison with 30% Acetic Acid<sup>a</sup>

Compounds	[(MeO)Tr]BzDA-OH			[(MeO) <sub>2</sub> Tr]BzDA-OH			[(MeO) <sub>2</sub> Tr]BzDA-p 3', 5'-CIPh		
	Condition	Depur. <sup>†</sup>	Prod. <sup>‡</sup>	Condition	Depur. <sup>†</sup>	Prod. <sup>‡</sup>	Condition	Depur. <sup>†</sup>	Prod. <sup>‡</sup>
2% BSA in CHCl <sub>3</sub> /MeOH (7:3)	0°C t = 20 min	12%	88%	0°C t = 3 min	4%	96%	0°C t = 2.5 min	a) nil	100%
1% BSA in CHCl <sub>3</sub> /MeOH (7:3)	room temp. t = 3.5 min	20%	80%	room temp. t = 1.5 min	15%	85%	room temp. 45 sec	b) nil	100%
30% AcOH	room temp. 2 hr	55%	45%	room temp. 30 min	36%	64%	room temp. 30 min	15%	85%

<sup>a</sup> Yield was estimated on silica-gel plates.

<sup>†</sup> Depurinated product found.

<sup>‡</sup> Expected deprotected intact product found.

a After 2 hr just a trace of depurination; after 6 hr less than 3%.

b After 20 min depurination less than 3%.

on silica-gel TLC plates and their tendency to solidify. Thus its structural analysis was much easier. Condensation of this 3'-O-benzoyl-N-protected mono- or oligodeoxynucleoside with a 5'-dimethoxytrityl-N-protected mono- or oligodeoxynucleoside-3'-(p-chlorophenyl) phosphate (about equimolar amounts) was carried in the presence of benzenesulfonyltetrazole (2-3 molar equivalent based on 5) in anhydrous pyridine for 5-12 hr at room temperature. The reaction mixture was then decomposed with aqueous pyridine followed by extraction with chloroform which was washed with 0.1 M triethylammonium bicarbonate pH 7.5. The chloroform solution was then co-evaporated with toluene to a gum which was chromatographed on a silica-gel column with chloroform:methanol (1-10% v/v) as eluent. The fractions displaying a trityl positive color test on TLC were pooled, concentrated to dryness and further treated overnight with excess of β-cyanoethyl phosphate (pyridinium salt) in the presence of benzenesulfonyltriazole in anhydrous pyridine solution. After the usual work-up pure fully protected oligonucleotide was obtained after silica-gel chromatography. The trityl group was now removed by treating with 2% chloroform

## MODIFIED TRIESTER APPROACH

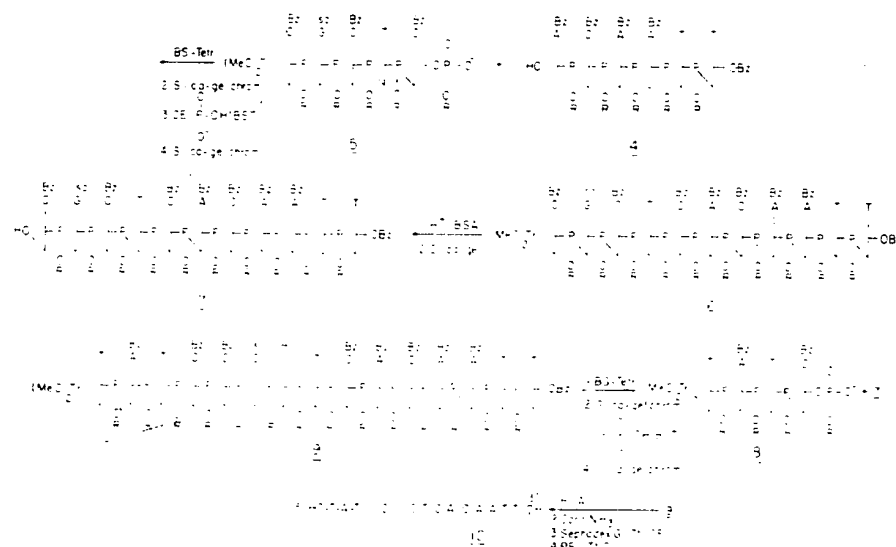


Figure 3. Synthesis of fully protected pentadecanucleotides of the defined sequence by the improved triester approach.

solution of benzenesulfonic acid at 0° and after the completion of the reaction the chloroform solution was washed with 5% sodium bicarbonate solution and water. The reaction conditions, isolated yields of the triester intermediates and yields of diester products after deblocking for various oligonucleotides appears in Table II.

#### Removal of the Protecting Groups

In our earlier studies<sup>1,16</sup> alkali labile protecting groups such as 3'-O-acetyl and p-chlorophenyl on the phosphotriester functions were removed by 0.1 N sodium hydroxide-dioxane-water treatment for 3-6 hr at room temperature, N-acetyl groups with concentrated ammonia at 50° and finally 5'-O-dimethoxytrityl groups with 80% acetic acid at room temperature. During these steps, we generally detected various impurities such as degradation products of the oligonucleotides, oligonucleotides containing 3'-3' phosphodiester bonds, cyclic dinucleotides<sup>4</sup>, unknown faster moving compound due to the degradation of the cytosine ring<sup>17</sup> and depurinating products. Recently we have observed that treatment with concentrated ammonia at 50°C for

4-6 hr removed phosphotriester. In the presence of a 2% solution followed by 1 hr. After chromatography fractions were pooled and cellulose fractionation plates.

The plates containing PEI-Cellulose concentrate-7 modification (up to 200 long column compounds) Characterized

In the oligomer analysis of the reaction of t and [γ-<sup>32</sup>P] diesterase intermediate the intermediate electrophoretic charge number of the number shift value interpreted adjacent maps for

4-6 hr removed the p-chlorophenyl group from the internucleotidic phosphotriester function with minimum formation of side products. In the present studies the dimethoxytrityl group was removed with a 2% solution of benzenesulfonic acid in chloroform at 0° followed by treatment with concentrated ammonia at 50°C for 4-6 hr. After removal of ammonia, the concentrated solution was chromatographed on Sephadex G-75 (superfine) column. The fractions containing the desired phosphodiester compound were pooled and further fractionated on a polyethylenimine-PEI-cellulose TLC plate as described below.

#### Fractionation of Synthetic Oligonucleotides on PEI-Cellulose TLC Plates

The final purification of the desired oligonucleotides containing 3'-5' phosphodiester functions were carried out on PEI-Cellulose TLC UV 254 plates<sup>6</sup> with eluent containing different concentrations of lithium chloride-7 M urea at 60°C or lithium acetate-7 M urea (pH 3.5) at room temperature. This is a modification of an earlier procedure<sup>18</sup>. A preparative scale (up to 200 Å<sub>260</sub>) separation of oligonucleotides up to 20 bases long could be achieved in 4 hr. The relative mobility of various compounds are given in Table III.

#### Characterization of Oligonucleotides by Two-Dimensional Procedure

In the present studies the base sequence of each synthetic oligomer was determined and confirmed by using the mobility shift analysis of Tu et al.<sup>12</sup>. This involves the labeling by phosphorylation of the 5'-hydroxyl group with polynucleotide kinase enzyme and [ $\gamma$ -<sup>32</sup>P] ATP followed by controlled snake venom phosphodiesterase digestion to obtain a sequential population of all the intermediate oligonucleotides. The total mixture containing all the intermediate products was fractionated by the two-dimensional electrophoresis-homochromatography system<sup>13</sup> which was based upon the charge and size difference of each oligonucleotide. The number of radioactive spots on the two-dimensional maps denote the number of bases in a given oligonucleotide and the mobility shift value<sup>12</sup> between the neighboring nucleotides could be interpreted to assign the particular base difference between adjacent oligonucleotides. Figure 4 shows the two-dimensional maps for the sequence analysis of P<sup>32</sup> (CGGATACAATT) and Figure 5

Table II. Reaction conditions and yields of various deoxyribonucleotides using benzenesulfonyltetrazole as the condensing reagent

[illegible]

Table 11 (contd.)

Prepolymer composition (mmol)	5-Hydroxyl component (mmol)	Condensing reaction time (hr)	Product (Yield %)	Deblocked product (Yield %)
100	100	1	100	100
100	100	2	100	100
100	100	3	100	100
100	100	4	100	100
100	100	5	100	100
100	100	6	100	100
100	100	7	100	100
100	100	8	100	100
100	100	9	100	100
100	100	10	100	100
100	100	11	100	100
100	100	12	100	100
100	100	13	100	100
100	100	14	100	100
100	100	15	100	100
100	100	16	100	100
100	100	17	100	100
100	100	18	100	100
100	100	19	100	100
100	100	20	100	100
100	100	21	100	100
100	100	22	100	100
100	100	23	100	100
100	100	24	100	100
100	100	25	100	100
100	100	26	100	100
100	100	27	100	100
100	100	28	100	100
100	100	29	100	100
100	100	30	100	100
100	100	31	100	100
100	100	32	100	100
100	100	33	100	100
100	100	34	100	100
100	100	35	100	100
100	100	36	100	100
100	100	37	100	100
100	100	38	100	100
100	100	39	100	100
100	100	40	100	100
100	100	41	100	100
100	100	42	100	100
100	100	43	100	100
100	100	44	100	100
100	100	45	100	100
100	100	46	100	100
100	100	47	100	100
100	100	48	100	100
100	100	49	100	100
100	100	50	100	100
100	100	51	100	100
100	100	52	100	100
100	100	53	100	100
100	100	54	100	100
100	100	55	100	100
100	100	56	100	100
100	100	57	100	100
100	100	58	100	100
100	100	59	100	100
100	100	60	100	100
100	100	61	100	100
100	100	62	100	100
100	100	63	100	100
100	100	64	100	100
100	100	65	100	100
100	100	66	100	100
100	100	67	100	100
100	100	68	100	100
100	100	69	100	100
100	100	70	100	100
100	100	71	100	100
100	100	72	100	100
100	100	73	100	100
100	100	74	100	100
100	100	75	100	100
100	100	76	100	100
100	100	77	100	100
100	100	78	100	100
100	100	79	100	

Table II (contd.)

5' Protected compound	5'-Hydroxyl compound	Condensing reagent	Reaction time (hr)	Product (yield %)	Released product (yield %)
HYDROLYSIS OF 5'-HYDROXY-3'-PHOSPHORYL-NUCLEOTIDES					
[10Me <sub>2</sub> ] <sub>2</sub> p[phosphoryl]GpCp (0.15)	5'-GMP (0.15)	0.6	0.5	[10Me <sub>2</sub> ] <sub>2</sub> p[phosphoryl]GpCp (74)	
[10Me <sub>2</sub> ] <sub>2</sub> p[phosphoryl]GpCp (0.15)	5'-GMP (0.15)	0.11	1	[10Me <sub>2</sub> ] <sub>2</sub> p[phosphoryl]GpCp (74)	dp-A-BU-BU-G-BU (80)
[10Me <sub>2</sub> ] <sub>2</sub> p[phosphoryl]GpCp (0.15)	5'-GMP (0.15)	0.15	1	[10Me <sub>2</sub> ] <sub>2</sub> p[phosphoryl]GpCp (74)	dp-A-T-BU-G-BU (78)
[10Me <sub>2</sub> ] <sub>2</sub> p[phosphoryl]GpCp (0.15)	5'-GMP (0.15)	0.9	1	[10Me <sub>2</sub> ] <sub>2</sub> p[phosphoryl]GpCp (74)	dp-A-T-T-G-BU (82)
1. DISTRIBUTION OF 5'-HYDROXY-3'-PHOSPHORYL-NUCLEOTIDES					
[10Me <sub>2</sub> ] <sub>2</sub> p[phosphoryl]GpCp (0.15)	5'-GMP (0.15)	0.75	1	[10Me <sub>2</sub> ] <sub>2</sub> p[phosphoryl]GpCp (74)	
[10Me <sub>2</sub> ] <sub>2</sub> p[phosphoryl]GpCp (0.15)	5'-GMP (0.15)	0.27	12	[10Me <sub>2</sub> ] <sub>2</sub> p[phosphoryl]GpCp (74)	dp-G-G-G-A-T-BU (60)
[10Me <sub>2</sub> ] <sub>2</sub> p[phosphoryl]GpCp (0.15)	5'-GMP (0.15)	1.2	1	[10Me <sub>2</sub> ] <sub>2</sub> p[phosphoryl]GpCp (74)	
[10Me <sub>2</sub> ] <sub>2</sub> p[phosphoryl]GpCp (0.15)	5'-GMP (0.15)	0.45	12	[10Me <sub>2</sub> ] <sub>2</sub> p[phosphoryl]GpCp (74)	dp-G-A-G-G-T-T-G-T (70)

Abbreviations are as suggested by the IUPAC-IUB. Hydrolysis 2, 40-22 (1970). A phospholust. linkage is represented by hyphen and phospholust. linkage is represented by 1° symbol. Each internal internucleotide phosphate is protected with p-chlorophenyl group (CIPh).

## Nucleic Acids Research

Table III.  $R_f$  Values<sup>a</sup> of Deoxyribonucleotides on PEI-Cellulose  
TLC Plates

Oligonucleotides containing phosphodiester groups	Solvent system	
	E (pH 8.0)	F (pH 3.5)
A-A-T-T-G-T	2.35	6.50
A-A-B-U-B-U-G-B-U	2.20	5.60
A-A-T-B-U-G-B-U	2.25	6.55
A-A-T-T-G-B-U	2.30	6.50
A-A-T-T-C-A-A-T-I-G-T	1.60	5.30
C-G-C-T-C-A-C-A-A-T-T	1.40	5.40
T-A-T-C-C-G-C-T-C-A-C-A-A-T-T	0.46	4.9
C-G-G-A-T-A-A-C-A-A-T-T	1.0	5.30
G-A-G-C-T-G-G-A-T-A-A-C-A-A-T-T	0.30	4.5
C-T-C-A-C-A-A-T-T	2.00	5.8
C-C-G-C-T-C-A-C-A-A-T-T	1.05	5.30
G-A-G-C-G-C-T-C-A-C-A-A-T-T	0.41	4.8
C-G-G-C-T-C-A-C-A-A-T-T	0.90	5.4
G-A-G-C-G-C-T-C-A-C-A-A-T-T	0.40	4.3
C-C-G-G-A-T-C-C-G-G	0.9	5.0
A-C-A-A-G-C-T-T-G-T	1.2	5.2

a) All  $R_f$  values are with respect to yellow dye.

for P<sup>32</sup> (CGGCTCACAATT). These maps unambiguously confirmed the entire sequence from the mobility shifts as mentioned above.

### CONCLUDING REMARKS

Thus the present studies have clearly demonstrated that these improvements in condensing agents, deblocking conditions and purification procedures have increased the effectiveness of the triester approach. It is a rapid method for the synthesis of long oligonucleotides of defined sequence in high yields.

### EXPERIMENTAL SECTION

#### General Methods and Materials

Thymidine, deoxyadenosine and deoxycytidine (Calbiochem), deoxyguanosine (Nutritional Biochemical Corp.), 5-bromodeoxyuridine, 1(H)-tetrazole, triisopropylbenzenesulfonyl chloride, mesitylenesulfonyl chloride, benzenesulfonyl chloride, *s*-cyanoethylphosphate, benzenesulfonic acid, monomethoxytrityl and

Figure

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N-benzo  
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Solvent

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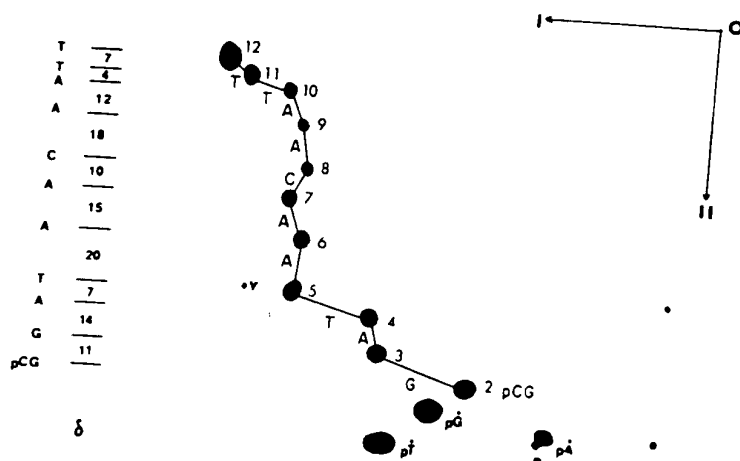


Figure 4. Two-dimensional fingerprinting of a partial snake venom phosphodiesterase digest of the dodecanucleotide p\*CGATAACAATT.

dimethoxytrityl chloride (Aldrich), Avicel-cellulose TLC plates containing fluorescence indicator, polyethylenimine (PEI/UV<sub>254</sub>) and silica gel F<sub>254</sub> thin layer plates, silica-gel H grade for column chromatography (Brinkman) and cellogram strip (Kalex Scientific Co., N.Y.) were purchased commercially.

Snake venom phosphodiesterase was purchased from Worthington Biochemical Co. and purified before use. T<sub>4</sub>-polynucleotide kinase (20,000 units/mg) was purchased from Biogenics Research Co. (Chagrin Falls, Ohio).

Homo-Mix and DEAE-cellulose plates were purchased or prepared according to previously reported procedures<sup>12,20</sup>.

N-Benzoyl-5'-O-dimethoxytrityldeoxyadenosine and N-benzoyl-5'-O-dimethoxytrityldeoxycytidine<sup>21</sup> and fully protected deoxymononucleotides containing 3'-(2-chlorophenyl)-5-cyanoethyl phosphate group<sup>4</sup> were prepared by the published procedures.

#### Solvent Systems

Oligonucleotides containing phosphotriester groups were analysed on silica-gel F<sub>254</sub> TLC plates by developing in solvent A, chloroform-methanol (1-10% v/v); for oligonucleotides containing phosphodiester groups Avicel-cellulose TLC plates were developed in solvent systems B [isopropyl alcohol-concentrated ammonium

hydroxide-water (7:1:2)], C [isobutyric acid-1 M ammonium hydroxide-0.1 M EDTA (100:60:1.6)], and D [*n*-propyl alcohol-concentrated ammonium hydroxide-water (55:10:35)]. PEI/UV<sub>254</sub> TLC plates were developed in solvent E, 0.6 M LiCl-7 M urea-0.025 M Tris (pH 8.0) at 60°C, or F, 0.6 M LiOAc-7 M urea (pH 3.5) at room temperature.

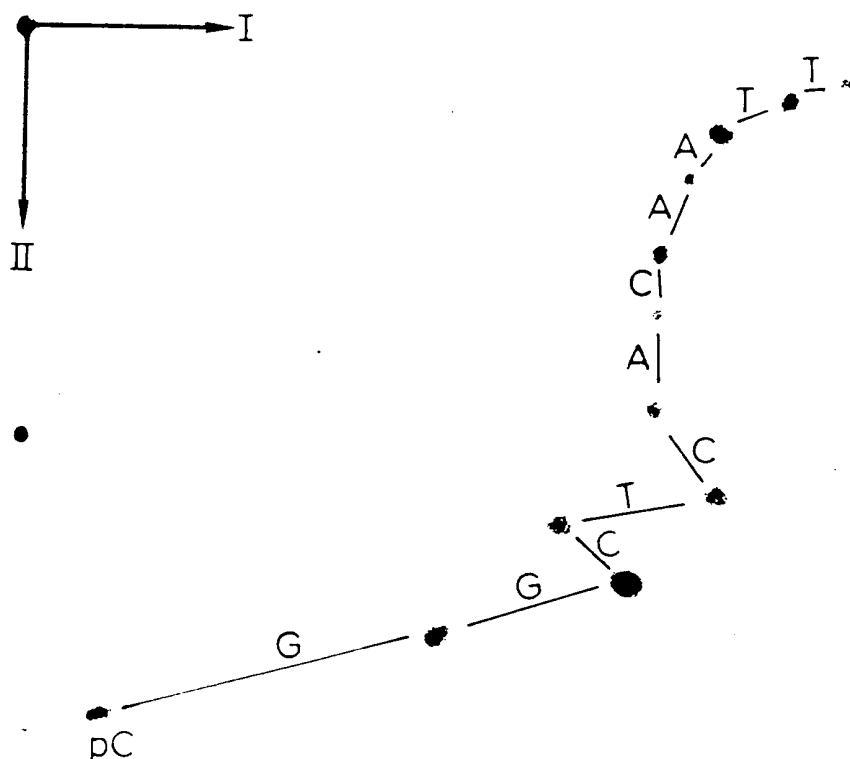


Figure 5. Two-dimensional fingerprinting of partial snake venom phosphodiesterase digest of the dodecanucleotide P\*CGGCTCACAATT.

#### General Method for the Preparation of Arylsulfonyltetrazoles

A dioxane (20 ml) solution of triethylamine (10.1 g, 0.1 mol) was added to a stirred suspension of arylsulfonyl chloride (0.1 mol) and 1 H-tetrazole (7.0 g, 0.1 mol) in dioxane (200 ml) at 5°C. After 2 hr the precipitate was filtered off and the dioxane solution was evaporated to dryness under vacuum. The crystalline residue was dissolved in chloroform (50 ml) which was

washe  
sulfa  
Recr;  
70-80  
benze  
remov  
dryne  
the c  
NMR s  
1-(Be  
Calcd  
C, 36  
(1H,  
1-(Me  
Calcd  
C, 47  
(1H,  
ortho  
1-(2,  
(Benz  
C, 57  
N, 16  
tetra  
3.14  
3'-O-  
solut:  
(16.3)  
After  
into :  
The c.  
anhyd:  
(free  
acid  
under  
crysta  
obtain  
276 nm  
C<sub>16</sub>H<sub>15</sub>



washed with water (2 x 20 ml), dried over anhydrous sodium sulfate, filtered and evaporated to provide a residue. Recrystallization from benzene produced the pure product in 70-80% yield. In the case of benzene sulfonyltetrazole the benzene solution was shaken with silica-gel for 1 hr and after removing silica-gel, the solution was evaporated under vacuum to dryness and recrystallized from benzene (60% yield). Each of the compounds were characterized by their elemental analysis and NMR spectra as detailed below (see Figure 2).

1-(Benzenesulfonyl)-tetrazole: m.p. 86-92° (Benzene). Anal. Calcd. for  $C_7H_6N_4O_2S$ : C, 36.36; H, 3.05; N, 28.27. Found: C, 36.50; H, 3.00; N, 28.24. NMR ( $CDCl_3$ , ppm from  $Me_4Si$ ) 9.25 (1H, s, CH in tetrazole); 8.0 (5H, m, aromatic H's).

1-(Mesitylenesulfonyl)-tetrazole: m.p. 108-119° (Benzene). Anal. Calcd. for  $C_{10}H_{12}N_4O_2S$ : C, 47.61; H, 4.79; N, 22.21. Found: C, 47.69; H, 4.84; N, 22.30. NMR ( $CDCl_3$ , ppm from  $Me_4Si$ ) 9.11 (1H, s, CH in tetrazole), 7.5 (2H, s, aromatic H's), 2.7 (6H, s, ortho  $CH_3$ ), 2.36 (3H, s, para  $CH_3$ ).

1-(2,4,6-Triisopropylbenzenesulfonyl)-tetrazole: m.p. 95-97° (Benzene-petroleum ether). Anal. Calcd. for  $C_{16}H_{24}N_4O_2S$ : C, 57.12; H, 7.19; N, 16.65. Found: C, 57.25; H, 7.20; N, 16.69. NMR ( $CDCl_3$ , ppm from  $Me_4Si$ ) 9.28 (1H, s, CH in tetrazole), 7.4 (2H, s, aromatic H's), 4.15 (2H, m, ortho  $CH_2$ ), 3.14 (1H, m, para  $CH$ ), 1.28 (18H, pseudoquartet  $CH_3$ ).

3'-O-Benzoyl 5-bromodeoxyuridine: To an anhydrous pyridine solution (60 ml) of 5'-dimethoxytrityl 5-bromodeoxyuridine (16.35 g, 30 mmol) was added benzoyl chloride (4.62 g, 33 mmol). After 2.5 hr at room temperature the reaction mixture was poured into ice water (500 ml) and extracted with chloroform (2 x 500 ml). The chloroform was washed with water (1 x 200 ml) and dried over anhydrous sodium sulfate, filtered and evaporated to dryness (free from pyridine) and the residue dissolved in 80% acetic acid (150 ml). After removing the acetic acid by evaporation under vacuum, the residue was coevaporated with toluene and on crystallization from ethanol, 3'-O-benzoyl, 5-bromouridine was obtained in 80% yield: m.p. 195-196°. UV spectrum (MeOH)  $\lambda_{max}$  276 nm ( $\epsilon$  11900);  $\lambda_{min}$  250 nm ( $\epsilon$  7500). Anal. Calcd. for  $C_{16}H_{15}N_2O_6Br$ : C, 46.7; H, 3.67; N, 6.81. Found: C, 46.6;

## Nucleic Acids Research

H, 3.59; N, 6.73.

Using the above 3'-O-benzoyl, N-isobutryldeoxyguanosine was also prepared in 70% yield: m.p. 145-147°. Anal. Calcd. for  $C_{21}H_{23}N_5O_6$ : C, 57.14; H, 5.25; N, 15.87. Found: C, 57.25; H, 5.38; N, 15.77.

### 5'-O-Monomethoxytrityl-N-isobutryldeoxyguanosine-3'-p-chlorophenyl-β-cyanoethyl Phosphate

Deoxyguanosine (50 mmol, 14.0 g) was made anhydrous by repeated (3 x 100 ml) evaporation under vacuo of its suspension in anhydrous pyridine. To the residue suspended in chloroform (300 ml) containing anhydrous pyridine (36 ml) was added dropwise isobutryl chloride (40 ml) in chloroform (200 ml) with stirring and cooling in ice bath. The reaction mixture became clear as the reaction proceeded and after 2 hr it was decomposed with water (100 ml). The organic layer was evaporated to dryness, the residue dissolved in ethanol (100 ml), treated with 2 N sodium hydroxide (100 ml) and kept at 0°C for 10 min. After neutralizing with excess Dowex resin (pyridinium form) and filtration the filtrate was evaporated to dryness in the presence of pyridine. The anhydrous pyridine solution (250 ml) was treated with monomethoxytrityl chloride (50 mM, 15.4 g), shaken for 20 hr, decomposed by adding cold water (100 ml) and after 20 min the solution was extracted with chloroform (3 x 200 ml). The combined organic layers were further washed with water (3 x 100 ml), evaporated to dryness in vacuo and then with toluene to provide a residue which was dissolved in chloroform (30 ml) and chromatographed on silica-gel column (500 g, 4 cm diameter) and eluted with chloroform-methanol (3.5 v/v). The product was isolated in 70% yield.

A mixture of p-chlorophenylphosphodichloridate (42 mmol), 1H-1,2,4-triazole (87 mmol) and triethylamine (87 mmol) was stirred in anhydrous dioxane (500 ml), first at 0°C for 10 min, and then at room temperature for 1 hr. The precipitate was filtered and the filtrate concentrated (to 250 ml) under reduced pressure, at  $\leq 23^\circ\text{C}$ . To this solution was added 5'-O-monomethoxytrityl, N-isobutryldeoxyguanosine (25 mmol), the reaction mixture kept at 20°C for 6 hr then β-cyanoethanol (35 mmol) was added and the reaction maintained overnight. The fully protected

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mononucleotide was isolated by silica-gel chromatography in 70-80% yield: m.p. 86-88°. UV spectrum (MeOH)  $\lambda_{\max}$  262 nm ( $\epsilon$  17000),  $\lambda_{\max}$  262 nm ( $\epsilon$  18500), and  $\lambda_{\min}$  250 nm ( $\epsilon$  15200). Anal. Calcd. for  $C_{44}H_{44}N_6O_{10}PCl$ : C, 59.95; H, 5.03; N, 9.08. Found: C, 59.90; H, 4.98; N, 8.97.

5'-O-Dimethoxytrityl-5-bromodeoxyuridine-3'-p-chlorophenyl- $\beta$ -cyanoethyl Phosphate

An anhydrous pyridine solution (10 ml) containing 5-bromodeoxyuridine (3.07 g, 10 mmol) and dimethoxytrityl chloride (3.5 g, 10.3 mmol) was shaken at room temperature for 3 hr. The reaction was stopped by the addition of methanol (1 ml) and this solution evaporated to dryness in vacuo at room temperature. To the residue was added chloroform (30 ml) and water (20 ml), partitioned, the chloroform phase was further extracted with water (2 x 10 ml), dried, evaporated and the residue taken up in chloroform (10 ml) and chromatographed on a silica-gel column (150 g, 4.5 cm diameter). The desired compound was isolated in 95% yield by eluting the column with chloroform-methanol (95.5:4.5 v/v).

A mixture of p-chlorophenylphosphodichloridate (5.6 mmol) 1H-1,2,4-triazole (11 mmole) and triethylamine (11 mmole) was stirred in anhydrous dioxane (50 ml) as described above. The phosphorylation and cyanoethylation reactions were carried out by adding 5'-O-dimethoxytrityl 5-bromouridine (4.6 mmol) followed 6 hrs later by  $\beta$ -cyanoethanol (4.6 mmol) as described above. The desired fully protected compound was isolated by silica-gel chromatography in 80% yield: m.p. 79-81° UV spectra (MeOH)  $\lambda_{\max}$  276 nm ( $\epsilon$  12000),  $\lambda_{\min}$  254 nm ( $\epsilon$  8600). Anal. Calcd. for  $C_{39}H_{37}N_3O_{10}PClBr$ : C, 54.9; H, 4.25; N, 4.92. Found: C, 54.8; H, 4.19; N, 4.90.

General Method for the Synthesis of Fully Protected Deoxyriboooligonucleotides

An anhydrous pyridine solution (5 ml per mmol of the nucleotidic component) containing 5'-O-dimethoxytritylnucleotide (or oligonucleotide)-3'-p-chlorophenyl phosphate plus an appropriate oligonucleotide containing a free 5'-hydroxyl and fully protected 3'-O-phosphate group (1.2 molar equivalent) was treated with benzenesulfonyltetrazole (3 molar equivalent relative to 3'-phosphodiester component) for 30 min to 12 hr at

## Nucleic Acids Research

room temperature. The reaction was then decomposed by addition of water (10 ml per g of the nucleotidic material) with cooling, followed by extraction with chloroform (100 ml per g of the nucleotidic material). The chloroform layer was washed with 0.1 N triethylammonium bicarbonate pH 7.5 (3 x 50 ml), water (1 x 50 ml), dried over anhydrous sodium sulfate, filtered, and under reduced pressure evaporated to a gum in the presence of excess toluene. The gum was dissolved in chloroform and chromatographed by column chromatography on silica gel in chloroform-methanol (1-10% v/v) solvent.

### Isolation of the Fully-Protected Deoxyribooligonucleotides

The fractions from the silica-gel chromatography (described above) exhibiting a positive trityl test on TLC were pooled, evaporated to dryness and treated with excess of  $\beta$ -cyanoethylphosphate (5 molar equivalent) in anhydrous pyridine solution (10 ml) in the presence of benzenesulfonyltriazole (3 molar equivalent based on  $\beta$ -cyanoethylphosphate) overnight at room temperature. After the usual work-up as described above, the required fully protected oligonucleotide was isolated by silica-gel chromatography. The reaction conditions and isolated yields are given in Table II.

### Deblocking of Monomethoxy- or Dimethoxytrityl Group from the Fully Protected Deoxyribooligonucleotides

The fully protected compound (100 mg) was dissolved in chloroform-methanol (1 ml, 7:3 v/v) containing 2% benzenesulfonic acid and kept at 0°C. The reaction was over in 20 min (monomethoxytrityl), 3 min (dimethoxytrityl), as checked by silica-gel TLC (chloroform:methanol 10:1 v/v). The reaction mixture was washed with a 5% sodium bicarbonate solution and then water. The chloroform layer was dried over anhydrous sodium sulfate, filtered and evaporated under reduced pressure. The residue was dissolved in chloroform (5 ml), applied to a silica-gel column (50 g, 4 cm diameter) and eluted with chloroform:methanol (97:3 v/v, 500 ml) at room temperature. By monitoring the fractions by silica-gel TLC in chloroform-methanol (9:1 v/v), the product was pooled and obtained in 90% yield.

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Complete Deblocking of the Fully Protected Deoxyribooligonucleotides

Removal of the dimethoxytrityl group from the fully protected compound (100 mg) was achieved by treatment with a 2% solution of benzenesulfonic acid in chloroform-methanol at 0°C for 3 min as described above. After work-up the residue was dissolved in concentrated ammonia (20 ml) containing pyridine (0.5 ml) to make an homogeneous solution and the sealed flask was heated at 50°C for 4-6 hr then concentrated to dryness in vacuo. The residue was dissolved in water (50 ml), extracted with ether (2 x 5 ml) and the aqueous layer was concentrated (1-2 ml) in the presence of pyridine and chromatographed on a column of sephadex G-75 (SF) K25/100. The fractions containing the desired compound were pooled, concentrated and further purified on polyethylenimine PEI/UV<sub>254</sub> TLC plate as described below.

Preparative Thin Layer Chromatography on Polyethylenimine-Cellulose (PEI/UV<sub>254</sub>) Plates

Before applying the samples, the PEI layers were predeveloped with methanol, dried and redeveloped with distilled water by the ascending technique. The plates were then dried and stored in the refrigerator. This treatment apparently reactivated the ion-exchange capacity and also removed a yellow discoloration material.

The samples of oligonucleotides (~100-150 Å<sub>260</sub> in 100 µl) were applied to the TLC plate as a narrow band along with dye marker<sup>22</sup> on each side of the sample. The plastic sheet was placed on a glass plate (20 x 20 cm) and a Whatman 3 MM paper was then clipped to the top of the plate and uniformly pressed against the PEI-plate by a plastic strip (2 cm x width of plate). The plate was first developed in water for 3 cm and then in solvent E, 0.6 M lithium chloride-7 M urea-0.025 M Tris (pH 8.0) at 60° till the blue dye was 2 cm from the top, or in solvent F, 0.6 M LiOAc-7 M urea (pH 3.5) at room temperature.

For recovery of materials, the wet plates were washed three times in methanol and then dried. The PEI-cellulose containing the desired band of product was removed and the compound was eluted with 2 M triethylammonium bicarbonate (pH 9.5)

## Nucleic Acids Research

### Characterization of Completely Unprotected Oligonucleotides Containing 3'-5' Phosphodiester Groups

#### I. PEI-Cellulose TLC

The mobilities of various oligonucleotides higher than hexamer compounds with respect to the yellow dye are given in Table III.

#### II. Sequence Analysis

#### Phosphorylation of Oligonucleotide with $T_4$ Polynucleotide Kinase and [ $\gamma$ - $^{32}$ P] ATP

The oligonucleotide (200 pmol) in 5  $\mu$ l of solution containing 66 mM Tris-HCl (pH 7.8), 6.7 mM  $MgCl_2$ , 15 mM dithiothreitol, and 66  $\mu$ M [ $\gamma$ - $^{32}$ P] ATP was incubated at 37° with 2-4 units of  $T_4$  polynucleotide kinase for 40 min. The reaction was stopped by the addition of excess EDTA. The 5'- $^{32}$ P labeled oligonucleotides were purified and desalted on a Sephadex G-50 column (fine) using 0.1 M triethylammonium bicarbonate (pH 8.0) as buffer.

#### Partial Venom Phosphodiesterase Digestion of $^{32}$ P-labelled Oligonucleotides<sup>23</sup>

The 5'-labeled oligonucleotide was digested at 37° in a 10  $\mu$ l solution containing 30  $\mu$ g of RNA carrier (partially hydrolyzed RNA), 50 mM Tris-HCl (pH 8), 5 mM  $MgCl_2$  and 0.2  $\mu$ g of venom phosphodiesterase. One-microliter samples were withdrawn at intervals of 5, 10, 20, 40, 60, 90, 120 and 180 min and blown into 50  $\mu$ l of 0.1 M EDTA. The combined digest was dried in a desiccator, dissolved in 2 to 5  $\mu$ l of water and applied to a cellulose-acetate strip (2.5 x 52 cm). Electrophoresis was carried out in pyridine-acetate buffer, pH 3.5, at 2500 volts for 30 to 60 min. The oligonucleotides were transferred onto a DEAE-cellulose thin layer plate. The plates were developed with water for 30 min and then in 2% partially hydrolyzed RNA containing 7 M urea (Homo-Mix) at 65° until the blue dye marker was within 1 inch of the top. The x-ray fingerprinting of two dodecamers is given in Figures 4 and 5.

#### ACKNOWLEDGMENT

We are grateful to Miss Suzanne Jackson, Carleton University, Ottawa, for her technical assistance.

\*To whom correspondence should be sent.

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**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicants: Dean L. Engelhardt et al.	)	
Serial No. 08/479,997	)	Group Art Unit: 1634
Filed: June 7, 1995	)	Exam'r: Scott W. Houtteman
Title: OLIGO- OR POLYNUCLEOTIDES, AND OTHER	)	
OTHER COMPOSITIONS COMPRISING PHOS-	)	
PHATE MOIETY LABELED NUCLEOTIDES	)	

Dearborn, Michigan

Honorable Commissioner of Patents and Trademarks  
The United States Patent and Trademark Office  
Washington, D.C. 20231

**DECLARATION OF DR. ANN SODJA**  
**(IN SUPPORT OF THE NON-OBVIOUSNESS OF THE INVENTION**  
**CLAIMED IN U.S. PATENT APPLICATION SERIAL NO. 08/479,997)**

I, Ann Sodja, hereby declare as follows:

1. I am presently Associate Professor in the Department of Biological Sciences at Wayne State University, Detroit, Michigan, having been appointed such in 1982. Under Dr. Paul K. Stumpf, my advisor, I received my doctorate in biochemistry from the University of California, Davis, California in 1974. My doctoral thesis was titled "Metabolism of Medium Chain Length Fatty Acids in Higher Plants." Earlier in 1964, I had received a Masters of Science in biochemistry from the Ohio State University, Columbus, Ohio. Prior to that, I had received an A.B. in chemistry with honors from Ursuline College, Cleveland, Ohio 1962. From 1974 through 1978, I was a post-doctoral fellow at California Institute of Technology in Pasadena, California, where I conducted research involving several gene families of *Drosophila melanogaster*. In particular, I worked on 5S RNA and tRNA genes, and techniques for their enrichment and mapping by electron microscopy. Dr. Norman Davidson was my post-doctoral mentor. It was also there that I initiated research on actin genes of *Drosophila melanogaster* and I continued that research for several years. In addition to presently being a tenured professor at Wayne State University, my professional appointments have included being appointed Associate Professor at WSU in 1982 and Assistant Professor at WSU in 1978. My academic background

Enz-5(D6)(C2)



and professional experience are listed on my curriculum vitae (CV) attached to this Declaration as Exhibit 1.

2. Among my honors and awards are the following: Charles Kettering Predoctoral Fellowship, 1962-1963; NIH Predoctoral Traineeship, 1963-1965; Max-Planck Fellowship for Visiting Scientists, 1967-1968; NSF Predoctoral Fellowship, 1969-1974; American Cancer Society Postdoctoral Fellowship, California Division, 1974-1976; Research Fellow in Chemistry, California Institute of Technology, 1976-1978; Career Development Chair Award, WSU, 1984; Presidential Excellence Award, WSU, 1986, 1987 & 1990; and Women of Wayne Staff/Faculty Recognition Award, honorable mention, 1989. I was also nominated by the students for the President's Award for Excellence in Teaching in 1990 and 1991. These and other professional honors and awards are listed on my CV (Exhibit 1).

3. I am the author of several scientific publications, including a number of published investigations dealing with the genetics and biochemistry of *Drosophila melanogaster* and its actin gene. These publications, including submitted publications and publications in preparation, number more than twenty and are listed on my CV (Exhibit 1). I have also published an equal number of abstracts which are listed on my CV (Exhibit 1). I have also authored five book reviews and other published materials as listed on my CV (Exhibit 1). In addition, I have presented thirty posters including eleven oral posters and nineteen poster presentations. I have given talks at more than two dozen seminars where I was invited to speak. These are also listed on my CV (Exhibit 1). Other professional experience including my professional memberships, fellowships and grants, faculty research and special awards, committee assignments, professional consultations, journal and editorial activities and other professionally related services are all listed on my CV (Exhibit 1).

4. Among my scientific publications is the Sodja and Davidson 1978 article published in Nucleic Acids Research (volume 5, pages 385-401) and titled "Gene Mapping and Gene Enrichment by the Avidin-Biotin Interaction: Use of Cytochrome-c as a Polyamine Bridge." A copy of my 1978 Nucleic Acids Research article is attached as Exhibit 2. In my investigation of the biochemistry and genetics of arthropods, including *Drosophila melanogaster*, spanning more than

Dean L. Engelhardt et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 3 [Declaration of Dr. Ann Sodja (In Support Of The Non-Obviousness Of The Invention Claimed In U.S. Patent Application Serial No. 08/479,997)]

three decades, I have examined nucleic acids, including DNA from a number of different species using a number of different and diverse formats. I am thoroughly familiar with nucleic acid detection formats and nucleic acid probe technology, having spent the better part of my professional career exploring their use as investigative tools for genetic analyses in arthropods and other species.

5. I have been engaged by Enzo Biochem, Inc. as a scientific consultant in order to review portions of the current prosecution of U.S. Patent Application Serial No. 08/479,997 ("Oligo- or Polynucleotides And Other Compositions Comprising Phosphate Moiety Labeled Nucleotides") that was filed on June 7, 1995. I am being compensated as a consultant by Enzo for this review and for making this Declaration. Included for my review were significant portions of the file wrapper for this application, including the original specification (hereinafter "the '997 specification"), the previously pending claims in this application (454-575), changes to independent claims (454, 482, 511 and 539)<sup>1</sup> to be submitted in a response (Amendment Under 37 C.F.R. §1.116) to the July 18, 2000 Office Action, and the latest composite set of claims (454-567) which will be pending in this application following the submission of the aforementioned Amendment After Final. A copy of the previously pending claims (454-575), the changes to the independent claims (454, 482, 511 and 539), and the latest composite set of claims (454-567) are attached to this Declaration as Exhibits 3, 4 and 5, respectively. I also understand that this Declaration will be submitted in connection with that aforementioned Amendment to be filed with the U.S. Patent and Trademark Office. I have also reviewed the July 18, 2000 Office Action as well as five other previous Office Actions issued on June 20, 1996, May 13, 1997, January 6, 1998, September 29, 1998 and February 3, 1999. I have also reviewed several papers filed in response to the aforementioned office actions. These papers include Applicants' June 23, 2000 Communication, their June 22, 2000 Second Supplemental Amendment, their June 20, 2000 Supplemental Amendment, their January 4, 2000 Amendment Under 37 C.F.R. §1.115, their January 19, 1999 Supplemental Response, their November 20, 1998 Amendment Under 37 C.F.R. §1.116, their July 6, 1998 Amendment Under 37 C.F.R. §1.115, their November 24, 1997 Amendment Under 37 C.F.R. §1.116, and their

<sup>1</sup> I understand that several dependent claims have also been amended. The affected dependent claims include 455, 459, 461, 466, 476, 480, 483, 487, 489, 494, 504, 508, 510, 512-531, 533, 535-559, 561 and 563-567. I have also reviewed the amendments to the dependent claims which will also be submitted in Applicants' January 18, 2001 Amendment Under 37 C.F.R. §1.116.

Dean L. Engelhardt et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 4 [Declaration of Dr. Ann Sodja (In Support Of The Non-Obviousness Of The Invention Claimed In U.S. Patent Application Serial No. 08/479,997)]

December 20, 1996 Amendment In Response To June 20, 1996 Office Action And Request For A Three Month Extension Of Time. I generally agree with the substance of Applicants' remarks and positions as set forth in these aforementioned responses, including those set forth in the Declaration of Dr. Dean L. Engelhardt In Support Of Adequate Description and Enablement that was submitted as Exhibit A to Applicants' November 27, 1997 Amendment Under 37 C.F.R. § 1.116 In Response To June 25, 1997 Office Action. I have also reviewed the Examiner Interview Summary Records dated November 3, 1998 and August 24, 2000. As the author, I am very familiar, of course, with the publication, Sodja and Davidson (1978) ["Gene Mapping and Gene Enrichment by the Avidin-Biotin Interaction: Use of Cytochrome-c as a Polyamine Bridge," Nucleic Acids Research 5:385-401 (1978)] (Exhibit 2), that in combination with another publication [Gohlke et al., U.S. Patent No. 4,378,458, issued on March 29, 1983, based on an application first filed on March 30, 1981] was cited in several office actions for obviousness by the U.S. Patent Examiner against the various pending claims in this application. A copy of the Gohlke '458 Patent is also attached to this Declaration as Exhibit 6.

6. I understand that in the latest July 18, 2000 Office Action claims 454-575 were rejected under 35 U.S.C. § 103 for being unpatentable over Gohlke et al., U.S. Patent No. 4,378,458 (Gohlke '458 Patent), issued on March 29, 1983, based on an application filed on filed March 20, 1981, in view of Sodja et al., Nucleic Acids Research 5(2):385-401 (1978) and further in view of applicant's admissions for reasons of record. I further understand that in the previous February 3, 1999 Office Action, the same claims were also rejected under 35 U.S.C. § 103 for being unpatentable over the Gohlke '458 Patent in view of Sodja et al. (1978) and further in view of applicant's admissions. In the February 3, 1999 Office Action (page 5), the Examiner stated:

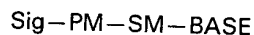
Applicant argues that the opening of the ring sugar in Sodja distinguishes Sodja from the claims of the current application. It is argued that the terminal nucleotide, with the open sugar, is outside the scope of the claims.

This argument is not persuasive. Sodja reads on the claimed invention because of the scope of the term "SIG" moiety. There is nothing in the limitation "SIG" which would exclude the terminal nucleotide, with the open ribose sugar from being part of the "SIG" moiety. The "terminal" nucleotide in the claimed product would be the second nucleotide from the end in the Sodja reference, which has a closed sugar ring.

Applicant argues that Gohlke does not teach labeling ribonucleotides and thus does not suggest the claimed DNA products. This argument is not persuasive. First, many of the claims of this case are not limited to DNA products but read on ribonucleotides. Second, it is Gohlke in view of Sodja which is the basis of the rejection. There is no evidence that Gohlke cannot be applied to Sodja for the expected benefit of generating other types of labeled oligonucleotides using the Gohlke labels.

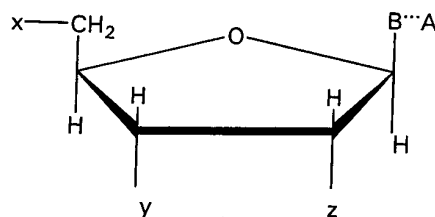
7. Based upon my review, I understand that the claimed invention is directed to detectable nucleic acid compositions, i.e., oligo- or poly(deoxyribo)nucleotides, comprising at least one modified nucleotide.

A. As set forth in amended claim 454 (Exhibit 4), one significant embodiment as I understand it is an oligo- or polydeoxynucleotide which is complementary to a nucleic acid of interest or a portion thereof. The oligo- or polydeoxynucleotide comprises at least one modified nucleotide having the formula



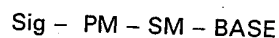
wherein PM is a phosphate moiety, SM is a sugar moiety and BASE is a base moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof. The PM is attached to SM, the BASE is attached to SM, and Sig is covalently attached to PM directly or through a chemical linkage. The element Sig comprises a non-radioactive label moiety which can be directly or indirectly detected when attached to PM or when the modified nucleotide is incorporated into the oligo- or polydeoxynucleotide or when the oligo- or polydeoxynucleotide is hybridized to the complementary nucleic acid of interest or a portion thereof.

B. As set forth in amended claim 482 (Exhibit 4) and as I understand it, another significant embodiment of the claimed invention is an oligo- or polydeoxyribonucleotide which is complementary to a nucleic acid of interest or a portion thereof. The oligo- or polydeoxyribonucleotide comprises at least one modified nucleotide having the structural formula:



In the structural formula, BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, and is attached to the 1' position of the pentose ring from the N1 position when BASE is a pyrimidine or from the N9 position when BASE is a purine or a deazapurine. The substituents x, y and z are selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate. Sig is covalently attached directly or through a chemical linkage to at least one phosphate selected from the group consisting of x, y, z, and a combination thereof. Sig comprises a non-radioactive label moiety which can be directly or indirectly detected when so attached to the phosphate or when the modified nucleotide is incorporated into the oligo- or polydeoxynucleotide or when the oligo- or polydeoxynucleotide is hybridized to the complementary nucleic acid of interest or a portion thereof.

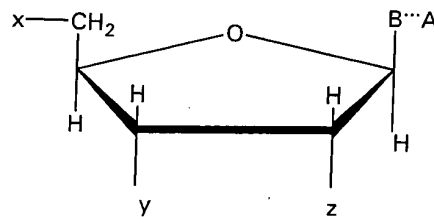
C. I understand that the claimed invention as set forth in amended claim 511 (Exhibit 4) is directed to an oligo- or polynucleotide which is complementary to a nucleic acid of interest or a portion thereof. The oligo- or polynucleotide comprises at least one modified nucleotide having the formula



wherein PM is a phosphate moiety, SM is a sugar moiety and BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof. PM is attached to SM, BASE is attached to SM, and Sig is covalently attached to PM directly or via a chemical linkage. Sig comprises a non-radioactive label moiety which can be directly or indirectly detected when attached to PM or when the modified nucleotide is incorporated into the oligo- or polynucleotide, or when the oligo- or polynucleotide is hybridized to the complementary nucleic acid of interest or

a portion thereof. When the oligo- or polynucleotide is an oligoribonucleotide or a polyribonucleotide, and when Sig is attached through a chemical linkage to a terminal PM at the 3' position of a terminal ribonucleotide, the chemical linkage in this oligo- or polynucleotide is not obtained through a 2',3' vicinal oxidation of a 3' terminal ribonucleotide previously attached to the oligoribonucleotide or polyribonucleotide.

D. I understand another embodiment as set forth in amended claims 539 (Exhibit 5) is an oligo- or polynucleotide which is complementary to a nucleic acid of interest or a portion thereof. The oligo- or polynucleotide comprises at least one modified nucleotide having the structural formula:



wherein BASE is a moiety selected from the group consisting of a pyrimidine, a purine and a deazapurine, or analog thereof, and wherein BASE is attached to the 1' position of the pentose ring from the N1 position when BASE is a pyrimidine or from the N9 position when BASE is a purine or a deazapurine. The elements x, y and z are selected from the group consisting of H—, HO—, a mono-phosphate, a di-phosphate and a tri-phosphate. Sig is covalently attached directly or through a chemical linkage to at least one phosphate selected from the group consisting of x, y and z, and a combination thereof. Sig comprises a non-radioactive label moiety which can be directly or indirectly detected when so attached to the phosphate or when the modified nucleotide is incorporated into the oligo- or polynucleotide, or when the oligo- or polynucleotide is hybridized to the complementary nucleic acid of interest or a portion thereof. Furthermore, when the oligo- or polynucleotide is an oligoribonucleotide or a polyribonucleotide and when Sig is attached through a chemical linkage to a terminal PM at the 3' position of a terminal ribonucleotide, the chemical linkage is not obtained through a 2',3' vicinal oxidation of a 3' terminal ribonucleotide previously attached to the oligoribonucleotide or

polyribonucleotide.

8. As Enzo's consultant and on its behalf, I am making this Declaration in support of the non-obviousness of the claims in this application which will be pending after submission of Applicants' Amendment Under 37 C.F.R. §1.116. To the extent that the subject matter is similar with those of the previously pending claims prior to submission of Applicants' Amendment, my remarks are applicable to those previously pending claims prior to the latest amendments as well.

9. Based upon my own training, background and experience, I would submit that at the time this application was first filed in June 1982, a person of ordinary skill in the art relevant to the subject matter being claimed, including nucleic acid modification, synthesis, hybridization and detection, would have possessed or could have been actively pursuing an advanced degree in organic chemistry and/or biochemistry. Such an ordinarily skilled person could also be at least approaching or ranging toward the level of a junior faculty member with 2-5 years of relevant experience, or would at least be a postdoctoral student with several years of experience. I consider myself to possess the level of skill and knowledge of at least a person of ordinary skill in the art to which the present application and invention pertains.

10. As a person of at least ordinary skill in the art to which the present invention pertains, it is my opinion and conclusion that the subject matter of claims 454-567 would not have been rendered obvious at the time the invention was made from a combined reading of the Gohlke et al. (U.S. Patent No. 4,378,458) in view of Sodja et al. [Nucleic Acids Research 5(2):385-401 (1978)] and further in view of applicant's admissions for reasons of record. My reasons why the invention set forth in claims 454-567 would have been non-obvious are set forth in the paragraphs below.

11. As the author of the cited Sodja and Davidson (1978) (Exhibit 2), I wish to point out that our work was intended to help us with electron microscopic gene mapping and gene enrichment of DNA:RNA hybrids. By coupling cytochrome-c to the oxidized 2', 3' terminus of RNA and attaching biotin labels to the coupled cytochrome-c, we found that electron microscopic gene mapping could be carried out efficiently with avidin-ferritin and avidin-polymethacrylate sphere labels. For

our gene mapping studies, Dr. Davidson and I used DNA and RNA from *E. coli* and *Drosophila melanogaster*. Examples of our results obtained with this method are shown by the electron micrographs in Figures 2 and 4 which are published in our 1978 paper (Exhibit 2) on pages 393 and 396, respectively. Furthermore, we found that gene enrichment was also efficiently obtained by buoyant banding of DNA:RNA-biotin:avidin-spheres in cesium chloride (CsCl) gradient. Results of our enrichment experiments for 5S rRNA from *Drosophila* DNA are presented in Table II on page 398 in my 1978 paper (Exhibit 2).

12. At the time when I was conducting experiments related to my 1978 paper, I was neither thinking nor intending to attach a detectable non-radioactive label to the terminus of RNA for the purpose of making a nucleic acid hybridization probe. Rather, after hybridizing the modified RNA with DNA, I was using large marker molecules, such as avidin-ferritin and avidin spheres, to produce more efficient gene mapping by electron microscopy and gene enrichment by cesium chloride gradient. In my work, we oxidized the free 2', 3' OH groups of RNA to the dialdehyde form using periodate. This is described in my 1978 paper both in the reaction scheme outlined on page 386 (no. 1) and in the MATERIALS AND METHODS Section on page 387 under Preparation and Purification of RNA-Cytochrome-c:

tRNA or 5S RNA were heated at 80° for 1-8 min in 1 mM NaAc buffer at pH 6.8, cooled, adjusted to 0.1 M NaAc buffer (pH 4.8) and treated with periodate as previously described (1). The amount of RNA used was 0.5 - 1 mg in 0.5 - 1 ml of reaction mixture.

The publication cited as (1) above is Broker et al., "Electron microscopic visualization of tRNA genes with ferritin-avidin:biotin labels," also in Nucleic Acids Research, 5(2):363-384 (1978). A copy of Broker et al. is attached to my Declaration as Exhibit 7.

13. As a person of ordinary skill in the art, I wish to point out that the periodate oxidation method used in my 1978 paper (Exhibit 2) and in Broker et al. (Exhibit 7) is applicable only to RNA which has two vicinal OH groups at the 3' and 2' positions. Other nucleic acids, including DNA, do not possess an OH group on the 2' position. Thus, the periodate oxidation method used in my 1978 paper (Exhibit 2) or Broker et al. (Exhibit 7) could not be used to attach a detectable non-



radioactive label to DNA as set forth, for example, in claims 454 and 482 (see amendments to independent claims (Exhibit 4) and composite set of claims (Exhibit 5) in this application.

14. As a person of ordinary skill in the art, it is my opinion and conclusion that the claims 511 and 539 in this application, which claims are drawn to an oligo- or polynucleotide, are outside of our 1978 paper (Exhibit 2) or Broker et al. (Exhibit 7). As set forth in Paragraph 7C and 7D above, the amendments to the independent claims (Exhibit 4) and the composite set of claims, claims 511 and 539 contain the proviso that

. . . provided that when said oligo- or polynucleotide is an oligoribonucleotide or a polyribonucleotide, and when Sig is attached through a chemical linkage to a terminal PM at the 3' position of a terminal ribonucleotide, said chemical linkage is not obtained through a 2',3' vicinal oxidation of a 3' terminal ribonucleotide previously attached to said oligoribonucleotide or polyribonucleotide.

Clearly, the fact that claims 511 and 539 eschew any and all chemical linkages which are obtained through a 2',3' vicinal oxidation of a terminal ribonucleotide, is significant because my 1978 paper relied exclusively on the vicinal oxidation of RNA using periodate. It is my opinion and conclusion that the subject matter of claims 511 and 539 would not have been taught or suggested to one of ordinary skill in the art at the time this application was first filed in June 1982 from reading my 1978 paper (Exhibit 2), taken with Gohlke's '458 Patent (Exhibit 6) and any of Applicants' admissions of record. As previously stated, the chemistry disclosed in my 1978 paper relied exclusively on vicinal oxidation of the free 2, 3' OH groups of RNA. The subject matter set forth in claims 511 and 539 clearly avoids such chemistry.

15. At the time that I conducted the experiments disclosed in my 1978 paper, I was concerned that the chemistry would not work with oligoribonucleotides (10 ribonucleotides or less) or very short polyribonucleotides. With such short pieces of RNA, I felt at the time that the addition of a large linker, such as cytochrome c, and a large biotin marker, might be too large in comparison to the length of the RNA such that steric hindrance would reduce, if not stymie hybridization between complementary RNA and DNA strands altogether.

Dean L. Engelhardt et al.

Serial No.: 08/479,997

Filed: June 7, 1995

Page 11 [Declaration of Dr. Ann Sodja (In Support Of The Non-Obviousness Of The Invention Claimed In U.S. Patent Application Serial No. 08/479,997)]

16. I understand that in the obviousness rejections made in both the February 3, 1999 and July 18, 2000 Office Actions, the Gohlke '458 Patent was cited as the primary reference, and my 1978 paper as the secondary reference. This was explained on page 5 in the February 3, 1999 Office Action:

it is Gohlke in view of Sodja which is the basis of the rejection. There is no evidence that Gohlke cannot be applied to Sodja for the expected benefit of generating other types of labeled oligonucleotides using the Gohlke labels.

It is my opinion and conclusion that even applying Gohlke's disclosed labels to my 1978 paper, one of ordinary skill in the art would not have arrived at the claimed invention in this application, as set forth in claims 454-567. As stated earlier, the chemistry used in my 1978 paper could not be applied to nucleic acids, such as DNA that lacked the 2' OH group otherwise found in RNA. Moreover, the claims drawn to the use of a terminal ribonucleotide as a modified nucleotide specifically avoid the vicinal oxidation and periodate chemistry described in my 1978 paper. Thus, using Gohlke's labels with the chemistry from my 1978 paper, one of ordinary skill in the art would not have arrived at the invention now claimed in this application. Nor, would such a person have had a reasonable expectation of success in reaching the present invention from a combined reading of Gohlke's 458 Patent, my 1978 paper and any statements made by the Applicants which are of record in this application.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that any such willful false statements may jeopardize the validity of the application or any patent issued thereon.

6/7/1/2001  
Date

Ann Sodja, Ph.D.  
Ann Sodja, Ph.D.

\* \* \* \* \*

Declaration.AS.1.17.01

Enz-5(D6)(C2)

**CURRICULUM VITAE**  
**ANN SODJA, Ph.D.**

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**Telephone Nos.:** (313) 577-2908 (Office)  
(313) 577-2925 (Laboratory)

**Birthplace:** Ljubljana, Slovenia  
Citizen of U.S.A.

**Education:**

Baccalaureate: A.B. in Chemistry with honors, Ursuline College, Cleveland, Ohio, 1962.

Graduate: M.S. in Biochemistry, the Ohio State University, Columbus, Ohio, 1964.

Doctoral: Ph.D. in Biochemistry, University of California, Davis, California, 1974.  
(Advisor: Dr. Paul K. Stumpf).

Postdoctoral California Institute of Technology, Pasadena, California, 1974-78.  
(Postdoctoral Mentor: Dr. Norman Davidson)

**Professional Appointments:** Tenured Associate Professor, Wayne State University, 1983.  
Associate Professor, Wayne State University, 1982.  
Assistant Professor, WSU, 1978.

**Professional Society Memberships:** AAAS, 1978-present.  
Sigma Xi, 1978-present.  
Association for Women in Science (AWIS),  
Detroit Chapter 1986 - present.  
AWIS, National Organization, 1988 - present.

**Honors/Awards:** National Honor Society and Dean's List  
(all throughout undergraduate education, 1958-62).  
A.B. cum laude, 1962.  
Charles Kettering Predoctoral Fellowship, 1962-1963.  
NIH Predoctoral Traineeship, 1963-1965.  
Max-Planck Fellowship for Visiting Scientists, 1967-1968.

NSF Predoctoral Fellowship, 1969-1974.  
American Cancer Society Postdoctoral Fellowship,  
California Division, 1974-1976.  
Research Fellow in Chemistry, California Institute of Technology,  
1976-1978.  
Career Development Chair Award, WSU, 1984.  
Presidential Excellence Award, WSU, 1986, 1987 & 1990.  
Women of Wayne Staff/Faculty Recognition Award,  
honorable mention, 1989.  
Nominated by the students for the President's Award for Excellence  
in Teaching 1990, 1991.

### Teaching Experience

Years at Wayne State: 20.  
Years at Other Colleges/Universities  
Ohio State University, 1962-64, Graduate T.A.  
University of California, Davis, 1970-71, Graduate T.A.

### Research

#### Fellowships:

##### Graduate:

Ann Sodja, Charles Kettering Predoctoral Fellowship, 1962-63,  
tuition plus stipend.  
Ann Sodja, NIH Predoctoral Traineeship, 1963-65, tuition plus stipend.  
Ann Sodja, NSF Predoctoral Fellowship, 1969-74, tuition plus stipend.

##### Postdoctoral:

Ann Sodja, Isolation of Duck Hemoglobin Genes Using Biochemical  
Gene Enrichment Techniques, 1974-76, American Cancer Society  
Fellow, California Division, approximate stipend.  
Ann Sodja, Cloning and characterization of tRNA and 5S RNA of  
*Drosophila melanogaster*, 1976-78, Research Fellow in Chemistry,  
California Institute of Technology, approximate stipend.

#### Grants:

##### Federal:

Ann Sodja, Co P.I., Contractile Protein Gene Expression During  
Development, 1981-85, NIH.  
Ann Sodja, Assoc. P.I., Contractile Protein Gene Expression During  
Development, following award periods:  
1982 (All these are subprojects in the University).  
1983 (wide proposal to NIH, Division of Research).  
1984 Resources, (Prof. Dunbar, PI).

1985.  
1986, (Each year's budget runs from 1/1-12/31  
of the same year).  
1987, 1988, 1989 & 1990.

Grants (All grants listed below are/were funded unless indicated otherwise.)

Federal:

Ann Sodja, Assoc. P.I., Actin Genes: Evolution of Their Structure and Regulation, GM08167, 1/1/91-1/1/95.  
1991 (This project was a subproject of the University wide proposal to the NIH, NIGMS (Prof. Dunbar, P.I.). Each years budget runs from 1/1-12/31 of that year.)  
1992, 1993 & 1994 (2/28/95).  
  
Ann Sodja, Acting P.I., DNA Fingerprinting and Paternity Assessment in Non-Human Primates, NSF BNS 88-18405, 7/31/90 - 7/31/92.  
Ann Sodja, Co P.I., Research Careers for Minority Scholars, NSF, W. Rolnick, PI (Physics), 1990-1995.  
NIH, NIGMS, MARC training program continuation renewal; Faculty Associate in the program. Prof. Jay, P.I. Funded for 6/1/92 - 5/3/97.  
Ann Sodja, Co P.I., A. Rosenspire, P.I., Proposal for Research Experience for Undergraduates, NSF, 1987 & 1988 (A/NF).

Private:

Ann Sodja, Characterization of Genes Coding for Actin in Drosophila melanogaster, 1979-80, Muscular Dystrophy Association (MDA).  
Ann Sodja, Characterization of Genes Coding for Actin in Drosophila melanogaster, 1980-81, MDA.  
Ann Sodja, Chromatin Structure During Actin Gene Expression, 1982-1983, MDA.  
Ann Sodja, Regulation of Actin Gene Expression, American Heart Association (MI.), 1/7/87-6/30/88.  
Sept. 9, 1987, Regulation of Actin Gene Expression, American Heart Association (MI), 1/7/88-6/30/90, (A/NF).

Grants Pending revisions/review/funding:

June 1, 1989, Actin Genes: Evolution of Their Structure and Regulation, Ann Sodja, PI, 33%, NIH, 1R01GM43785-01, (TDC), 4/1/90 - 3/31/95, (A/NF).  
December 1, 1989, Actin Genes: Evolution of Their Structure and Regulation, Ann Sodja, PI, 33%, NSF, DMB-9003727, (TDC), 7/1/90-6/30/93, (A/NF).  
March 22, 1991, Actin Gene(s) in Drosophila virilis, Ann Sodja, PI, 10%,

Research Award Women of Wayne Alumni Association, 1991-92,  
(A/NF).

October 14, 1991, Supplemental Research Equipment Fund, WSU, submitted  
jointly with Prof. Arking, (NF).

Grants in preparation:

New project on molecular biology of the mosquito.  
(No funding information available at this time).

Other Fellowships:

Grant-in-Aid Awards, WSU-ORSPS (the ultimate source of money is NIH,  
Biomedical Research Division.):

Ann Sodja, Characterization of Actin Genes in *Drosophila melanogaster*,  
1979-80.

Ann Sodja, Comparison of Actin Coding and Its Adjacent-Sequences in  
Evolution, 1980-81.

Ann Sodja, Chromatin Structure during Actin Gene Expression, 1981-82.

Ann Sodja, Molecular Comparison of Actin Genes in *Drosophila melanogaster* &  
*Musca Domestica*, 1984-85.

Ann Sodja, Coordinate Control of Actin Gene Expression, 1985-86.  
1985, on a noncompetitive basis.

August, 12, 1988, Evolution of Actin Genes and Regulation of Their Expression,  
1988-89 (A/NF).

Faculty Research Awards:

Ann Sodja, Regulation of Actin Gene Expression, 1987-88.

Special Awards:

Ann Sodja, Biochemical Characterization of Collagen/Tropocollagen, 1967-68,  
MaxPlanck Gesellschaft, (Visiting Scientist's Fellowship)

Ann Sodja, Developmental Actin Gene Expression, 1984-86, Career  
Development Chair Award (WSU).

Ann Sodja, Actin Gene Regulation in *Drosophila*, 1986-89, Molecular Biology  
Center (WSU).

Ann Sodja, Postdoctoral Fellow Position Presidential Excellence Award, 1986.

Ann Sodja, Received in matching funds a second postdoctoral fellowship position from  
the Center of Molecular Biology (CMB), (WSU), 1986.

Ann Sodja, Postdoctoral Fellowship position from CMB (WSU), 1987.

CMB Predoctoral Fellowship for Christopher Horak, 1986-88.

Ann Sodja, Travel Award from L.A. College and VPR's office, 1987.

Ann Sodja, President's Excellence Award, 1987.

Ann Sodja, Postdoctoral Fellowship position from CMB (WSU), 1988.

Ann Sodja, matching funds for publication costs.

CMB Predoctoral Fellowship for Xisojun Yan, 1988-1989, and 1989-1990.

CMB salary support for Baolin Wang, 9/15/88-3/31/89.

January 2, 1990, Molecular Characterization of Actin Gene(s) in *Drosophila virilis*, Ann Sodja, PI, 33%, WSU Presidential Excellence Award through Biological Sciences.

May 13, 1991, Actin Gene(s) in *Drosophila virilis*, Ann Sodja, PI, 30%, Biomedical Research Support, 1991-1992.

December 5, 1995-1996. Research Stimulation Fund Grant to initiate molecular investigation on mosquito from the Office of the Vice President for Research and in matching funds from the Department of Biological Sciences.

March, 1996, Arthropod-Borne and Infectious Diseases Lab (AIDL), Ft. Collins, CO. Award- to cover expenses for attendance of Biology Disease Vectors. External \$1,680 (MacArthur Foundation).

April, 1996, Research Development Award.

January 1999, Olfactory Receptors Guide Mosquitoes to their Blood Meal, Research stimulation grant, WSU.

## **Publications**

### Journal Articles Published (all years)

Taniuchi, H., C.B. Anfinsen, A. Sodja, The Amino Acid Sequence of an Extracellular Nuclease of *Staphylococcus aureus*, II. The Amino Acid Sequences of Tryptic and Chymotryptic Peptides, *J. Biol. Chem.*, 242:4735-4751 (1967).

Taniuchi, H., C.B. Anfinsen, A. Sodja The Amino Acid Sequence of an Extracellular Nuclease of *Staphylococcus aureus*, III. Complete Amino Acid Sequence, *J. Biol. Chem.*, 242:4752-4767 (1967).

Taniuchi, H., C.B. Anfinsen, A. Sodja, Nuclease-T: An Active Derivative of Staphylococcal Nuclease Composed of Two Noncovalently Bonded Peptide Fragments, *Proc. Nat. Acad. Sci.*, 58:1235-1242 (1967).

Harwood, J.L., A. Sodja, P.K. Stumpf, A.R. Spurr, On the Origin of Oil Droplets in Maturing Castor Bean Seeds, *Ricinus Communis*, *Lipids* 6:851-862 (1971).

Harwood, J.L., A. Sodja, P.K. Stumpf, Beta Hydroxylation of Fatty Acids by a Soluble Preparation from Maturing Avocado Mesocarp, *Biochem. J.* 130:1013-1022 (1972).

Sodja, A., P.K. Stumpf, Fat Metabolism in Higher Plants: Metabolism of Medium Chain Fatty Acids, *Lipids* 10:818-828 (1975).

- Yen, P.H., Sodja, A., Cohen, M. Jr., Conrad, S.E., Wu, M., Davidson, N., Ilgen, C., Sequence Arrangement of tRNA Genes on a Fragment of *Drosophila melanogaster* DNA Cloned in *E. coli*. *Cell* 11: 763-777 (1977).
- Hershey, N.D., Conrad, S.E., Sodja, A., Yen, P.H., Cohen, M. Jr., Davidson, N., Ilgen, C., Carbon, J., The Sequence Arrangement of *Drosophila melanogaster* 5S DNA Cloned in Recombinant Plasmids. *Cell* 11: 585-598 (1977).
- Sodja, A., Davidson, N., Gene Mapping and Gene Enrichment by the Avidin-Biotin Interaction: Use of Cytochrome-c as a Polyamine Bridge. *Nucleic Acids Research* 5: 385-401 (1978).
- Davidson, N., Fyrberg, E.A., Hershey, N.D., Kindle, K., Robinson, R.R., Sodja, A., Yen, P., Recombinant DNA Studies of DNA Sequence Organization Around Actin and tRNA Genes of *Drosophila* in "RNA-Polymerases, tRNA and Ribosomes: Their Genetics and Evolution," S. Osawa, H. Ozeki, H. Uchida and T. Yura, Eds., University of Tokyo, p. 279-295 (1980).
- Fyrberg, E.A., Kindle, K.L., Davidson, N. and Sodja, A., The Actin Genes of *Drosophila*: A Dispersed Multigene Family. *Cell* 19: 365-378 (1980).
- Sodja, A., Arking, R., Zafar, R.S., Actin Gene Expression During Embryogenesis of *Drosophila melanogaster*. *Develop. Biol.* 90: 363-368 (1982).
- Sodja, A., Rizki, R.M., Rizki, T.M., Zafar, R.S., Overlapping Deficiencies Refine the Map Position of the Sex-Linked Actin Gene of *Drosophila melanogaster*. *Chromosoma* 86: 293-298 (1982).
- Zafar, R.S., Sodja, A., Homology in the Actin Coding and Adjacent Sequences in two Widely Divergent Species. *Biochem. Biophys. Res. Comm.* 111:67-73 (1983).
- J. Papa Rao, Zafar, R.S., Sodja, A., Transcriptional Activity at the 3' End of the Actin Gene at 5C on the X Chromosome in *Drosophila melanogaster*. *Biochim. Biophys. Acta*, 950:30-44 (1988).
- Wang, B., and Sodja, A., Alternate Approach to Sequencing Double-Stranded Template DNAs. *BioTechniques* 10: 198-201 (1991).
- Winrow, M.A., and Sodja, A. Cloning, Initial Characterization of Several Actin Genes in the Parasitic Nematode, *Ascaris suum*. *Biochem. Biophys. Res. Comm.* 178: 578-585 (1991).
- Rao, J.P., and Sodja, A. Further Analysis of a Transcript Nested Within Actin 5C Gene of *Drosophila melanogaster*. *Biochem. Biophys. Res. Comm.* 184:400-407 (1992).



Hadden, T.J., and Sodja A., An Oligogene Family Encodes Actins in the Housefly, Musca domestica, Biochem. Biophys. Res. Comm. 203:523-531 (1994).

Journal Articles (Submitted/In Press):

Hadden, T.J., and Sodja, A., A Housefly Actin Gene: Interspecies Conservation of Coding and Putative Regulatory Sequences.

Journal Articles in Preparation:

Wang, B.L., Liu, G., and Sodja, A. An Actin Gene of Drosophila virilis.

Sodja, A., Fujioka, H., Lemos, F.J.A., Donnelly-Doman, M., and Jacobs-Lorena, M. (2000). The Induction of Actin Gene Expression in the Mosquito Midgut in Response to Blood Ingestion Correlates with Dramatic Changes of Cell Shape. Manuscript to be submitted shortly.

Abstracts Published:

Sodja, A., Hershey, N.D., Conrad, S.E., Davidson, N., Ilgen, C., Carbon, J., Mapping Drosophila 5S Genes in Several Recombinant Plasmids. J. SUPRAM ST1977 (S1):53 Abstract from the ICN-UCLA Symposia on the Molecular Approaches to Eucaryotic Genetic Systems (1977).

Sodja, A., Isolation and Characterization of Actin Genes in Drosophila melanogaster. In Gene Structure and Expression (Ohio State University Biosciences Colloquia, No. 6), D.H. Dean, L.F. Johnson, P.C. Kimball, R.S. Perlman, Eds., The Ohio State University Press (1980).

Sodja, A., Zafar, R.S., Arking, R., Differential Actin Gene Expression in Drosophila melanogaster. Abstract of the Cleveland Symposium on Macromolecules, International Congress on Recombinant DNA, Elsevier Publishing Co., p. 32 (1981).

Sodja, A., Zafar, R.S., Mildner, A.M., Actin Gene Expression During Development of Drosophila melanogaster. Abstract of the Symposium Presented at the 149th National Meeting of the American Association for the Advancement of Science, Detroit, Michigan, AAAS Abstracts, p. 42 (1983).

Sodja, A., Zafar, R.S., Developmentally Linked transcription at the Sex-Linked Actin Gene in Drosophila melanogaster. Abstract of the Poster Session Presented at the UCLA Symposium on Molecular Biology of Development held in April, 1984 at Steamboat, Colorado. Abstract in J. Cell. Biochem., Supplement 8B, p. 50 (1984).

Sodja, A., Mildner, A.M., Elsenboss, L., Actin Genes in Different *Drosophila* Species, abstract of the poster session presented at UCLA symposium on Molecular Biology of Muscle Development held in March, 1985 in Park City, Utah. Abstract in J. Cell. Biochem. Supplement 9B, p. 53, 1985.

Winrow, M.A., Martin, P.E., Sodja, A., Preliminary Study of Actin Genes in *Ascaris suum*, abstract of a poster presented at the 13th Annual MBRS Symposium held in Miami, Florida, April, 1985. Abstracts #104, p. 18.

Elsenboss-Pena, L., Sodja, A., Only 3 Genes Encode Actins in *Drosophila virilis*, abstract of the poster presented at UCLA Symposium on Molecular Entomology, J. Cell. Biochem. Supplement 10C, p. 72 (1986).

Winrow, M.A., Martin, P.E., Sodja, A. A Study of Actin Genes in *Ascaris suum*, abstract of the poster presented at UCLA Symposium on Molecular Biology of Invertebrate Development, J. Cell. Biochem. Supplement 11C, p.31, 1987.

Zafar, R.S., J. Papa Rao, Sodja, A. A Unique Configuration at the 3' End of Actin Gene of *Drosophila melanogaster*, 18th FEBS meeting Abstract TU 2.2.5, p. 24, 1987.

Sodja, A., J. Papa Rao, Zafar, R.S. An Actin Associated 3' UTR in *Drosophila* Contains a Transcriptional Unit. UCLA Symposium on Molecular Biology of RNA, J. Cell. Biochem., 12C, N720, 1988.

Hadden, T.J., Sodja, A. The Actin Genes of *Musca Domestica*. 4th International Congress of Cell Biology, Abstracts, #8.4.26, p. 303, 1988.

J. Papa Rao, Zafar, R.S., Sodja, A. A Transcriptional Unit Within a *Drosophila* Actin Associated 3'UTR, XVIth International Congress of Genetics, Abstracts, #31.12.4, p. 63, 1988.

J. Papa Rao, Zafar, R.S., Sodja, A. Overlapping Transcripts of an Actin Gene in *Drosophila melanogaster*, 18th Annual Cardiovascular Research Forum, Abstracts, p. 65, 1988.

Jackson, N., Hadden, T.J., Sodja, A. The Actin Genes of *Musca domestica*, abstract of a poster presented at the 16th Annual NIH-MBRS Symposium, Los Angeles, California, Oct. 13 - 15, 1988.

McMullen, D.M., Winrow, M.A., Sodja, A., Actin Genes in *Ascaris suum*, 17th Annual NIH-MBRS Symposium, Houston, Texas, Abstract 73, p.24, 1989.

Hadden, T., Kim K., Sodja, A., Molecular Characterization of Actin Genes in Musca domestica, 32nd Annual Drosophila Research Conference, Chicago, Illinois, Abstract 23.149, p. 56, 1991.

Sodja, A., Hadden, T.J., Evolutionary Conservation of a Serum Response Element Between Human and Housefly Genes, Human Genome '92 International Conference, Nice, France, #132, p. 64, 1992.

Sodja, A., Hadden, T.J., Detailed Characterization of a Putative Muscle-Specific Gene in the Housefly, Musca domestica, 34th Annual Drosophila Research Conference, San Diego, California, Abstract 42A, p. 46, 1993.

Sodja, A., Hadden, T.J., Conservation of Actin Gene Regulatory and Structural Features Between Two Dipterans. 21st Annual NIH-NIGMS Minority Programs Symposium, Abstract 163, p. 53, 1993.

Sodja, A., Hadden, T.J., Analysis of Dipteran Actin Genes Suggests Conservation of Regulatory Elements Between Vertebrates and Invertebrates. Keystone Symposia on Molecular Biology of Muscle Development, J. Cell. Biochem. Supplement 18D, Abstract W 357, p. 522, 1994.

#### Book Reviews and Other Published Materials

Davidson, N., Fyrberg, E.A., Hershey, N.D., Kindle, K., Robinson, R.R., Sodja, A., Yen, P. 1980. Recombinant DNA studies of DNA Sequence Organization Around Actin and tRNA Genes of Drosophila, In RNA polymerase, tRNA and ribosomes: Their Genetics and Evolution. University of Tokyo, P. 279-295.

NOTE: *This book resulted from an International Symposium on Genetics and Evolution, in Tokyo, 1979 at which this paper was presented.*

Sodja, A., Effect of Tetracycline on Phagocytosis of Polystyrene Spheres by Polymorphonuclear Leucocytes of Guinea Pigs, M.S. Dissertation, 1964.

Sodja, A., Metabolism of Medium Chain Length Fatty Acids in Higher Plants, Ph.D. Dissertation, 1974.

Together with my T.A., Erle Robertson, planned experiments and compiled a lab manual for the Howard Hughes Summer Institute Program on Molecular Biology of the Gene, Winter semester 1991. The actual course offering was in Summer, 1991.

Preparation of the Biochemistry laboratory manual for the Howard Hughes Summer Institute Program in Biochemistry, offered in Summer 1994.

Book Review of Biochemistry, 2nd edition, by D. Voet and J.G. Voet, for the International Biodeterioration and Biodegradation, 37:233-235 (1996).

Papers/Posters Presented

*Oral Presentations*

The National Drosophila Symposium, University of Indiana, Bloomington, Indiana, May 17-19, 1979.

The 6th. Annual College of Biological Sciences Colloquium, Ohio State University, Columbus, Ohio, 6-8 Sept. 1979.

The International Workshop on Molecular Biology of *Drosophila melanogaster*, Crete, Greece, August 1981 (By invitation).

The 22nd National Conference on Drosophila Genetics, Chicago Ill., April 1981.

Invited speaker: International Congress on Recombinant DNA, Cleveland, Ohio, June, 1981.

Midwestern Drosophila Conference, Allenton Park, Illinois, Oct. 1982 and 1985.

Invited symposium speaker: 149th National Meeting of the AAAS, Detroit, Michigan, 1983, (By invitation).

Invited symposium speaker: 18th FEBS Meetings, Yugoslavia, June 28 - July 3, 1987, (By invitation).

Invited speaker: WSU CLL workshop on Biotechnology, Jan. 30, 1988.

Invited participant: Keystone Symposium: Fundamental Mechanisms of Transcription, Copper Mountain, Colorado, March 28-April 3, 1992.

Invited speaker: Vector Encounter, Case-Western Reserve University, Cleveland, Ohio, June 25-26, 1999.

*Poster Presentations*

Sodja, A., Hershey, N.D., Conrad, S.E., Davidson, N., Ilgen, C., Carbon, J., ICN-UCLA Symposium on the Molecular Approaches to Eucaryotic Genetic Systems, Park City, Utah, March 1977.

Sodja, A., 1980. Ohio State University Biosciences Colloquia, No. 6, Ohio State University, Ohio, Sept. 1980.

Sodja, A., Zafar, R.S., Cetus UCLA Symposium on the Molecular Biology of Development, Steamboat Springs, Colorado, April 1984.

Sodja, A., Mildner, A.M., Elsenboss, L. UCLA Symposium on Molecular Biology of Muscle Development. Park City, Utah, March 1985.

Winrow, M.A., Martin, P.E., Sodja, A., 13th Annual MBRS Symposium, Miami, Florida, April, 1984.

Elsenboss-Pena, L., Sodja, A., UCLA Symposium on Molecular Entomology, Steamboat Springs, Colorado, April 1986.

Hadden, T. J., Sodja, A., Actin Genes in Two Distantly Related Dipterans, The National Drosophila Meetings, Asilomar, California, April 1986.

Elsenboss-Pena, L., Sodja, A., AWIS, Detroit Science Center, Jan. 31 -Feb. 1, 1987.

Winrow, M.A., Martin, P.E., Sodja, A., UCLA Symposium on Molecular Biology of Invertebrate Development, in Park City Utah, March 15-22, 1987.

Hadden, T.J., Sodja, A., 4th International Congress of Cell Biology, Montreal, Canada, August 14-20, 1988.

J. Papa Rao, Zafar, R.S., Sodja, A., XVIth International Congress of Genetics, Toronto, Canada, August 20-27, 1988.

J. Papa Rao, Zafar, R.S., Sodja, A., 18th Annual Michigan Cardiovascular Research Forum, American Heart Association of Michigan, University of Michigan, Ann Arbor, Michigan, September 15, 1988.

Jackson, N., Hadden, T.J., Sodja, A., 16th Annual NIH-MBRS Symposium Program, Los Angeles, CA, Oct. 13 - 15, 1988.

McMullen, D.M., Winrow, M.A., Sodja, A., 17th Annual NIH-MBRS Symposium, Houston, Texas, Oct. 4-8, 1989.

Hadden, T., Kim K., Sodja, A., 32nd Annual Drosophila Research Conference, Chicago, Illinois, March 20-24, 1991.

Sodja, A., Hadden, T.J., Human Genome '92, 4th International Conference, Nice, France, Oct. 14-17, 1992.

Sodja, A., Hadden, T.J., 34th Annual Drosophila Research Conference, San Diego, California, Mar. 31-Apr. 4, 1993.

Sodja, A., Hadden, T.J., 21st Annual NIH-NIGMS Minority Programs Symposium, Atlanta, Georgia, Nov. 3-7, 1993.

Sodja, A., Hadden, T.J., Keystone Symposia on Molecular Biology of Muscle Development, Snowbird, Utah, April 11-17, 1994.

NOTE: All of the poster sessions, except for the one presented at Nat'l Drosophila Meetings, have published abstracts listed with full titles and other information under D (Abstracts Published). The oral presentations at the International Congress on Recombinant DNA (Cleveland, Ohio), at the AAAS meetings (Detroit, Mi.) and also at the 18th FEBS meetings have abstracts.

#### **Invited Seminars Presented**

University of California, Los Angeles, 1976.  
Michigan State University, East Lansing, Michigan, 1976.  
University of California, Los Angeles, 1976.  
Michigan State University, East Lansing, Michigan, 1976.  
Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland, 1977.  
Centre of Biochemistry and Molecular Biology, CNRS, Marseille, France, 1977.  
Biozentrum, Basel, Switzerland, 1977.  
University of Iowa, Iowa City, Iowa, 1977.  
University of Amsterdam, Amsterdam, Holland, 1977.  
University of Iowa, Iowa City, Iowa, 1977.  
Oakland University, Rochester, Michigan, 1979.  
Developmental Biology Interest Group, WSU, 1979 and 1983.  
University of Michigan, Ann Arbor, 1980.  
Case-Western Reserve University, Cleveland, Ohio, 1980.  
University of California, Los Angeles, CA, 1980.  
Ohio State University, Columbus, Ohio, 1980.  
University of Toledo, Toledo, Ohio, 1984.  
University of Detroit, Detroit, 1984.  
Wayne State University, Biochemistry Department, 1984.  
University of Ljubljana, Ljubljana, Yugoslavia, 1984.  
Illinois State University, Normal, Illinois, 1985.  
Oakland University, Rochester, Michigan, 1986.  
Stroh's Brewery Co., Detroit, MI, 1987.  
Wayne State University, Dept. of Physiology, 1987.  
Heidelberg College, Tiffin, Ohio, 1988.  
University of California, Los Angeles, 1993.

Case-Western Reserve University, Cleveland, Ohio, 1995.

## **Committee Assignments**

### *Community*

Judge at the Annual Southeastern Michigan Junior Science Fair and Humanities Symposium held at WSU, 1980 and 1981.

Judge for selecting presentations at this Fair in 1982.

Panel member on the NSF Visiting Professorship for Women, Student Center Bldg., WSU, 1986.

Poster presentation at the Detroit Science Center, Program organized by the Association for Women in Science (AWIS), Detroit Chapter, Jan. 30 -Feb. 1, 1987.

Colloquium speaker at AWIS Workshop on Extramural Funding, Farmington Hills Public Library, March 28, 1987.

As member of the National Association for Women in Science and past president of its Detroit area chapter, I am involved in activities which promote sciences as viable careers for women. Toward achieving its goals our chapter sponsors a number of activities such as a monthly public lecture given by a woman scientist; a weekly workshop at the Detroit Science Center for girl scouts (about 30-60 participants/workshop) and for the third consecutive year we published a calendar featuring women in Science (Nobel Prize laureates and nominees; the 1989 calendar featured scientists inducted into the Michigan Women's Hall of Fame). We have recently developed several fund raising functions: a fund raising banquet featuring Dr. Isabela Lugoski and her Nobel Prize laureate husband; sale of T-shirts and mugs as well as a "corporate letter" to industries interested to support AWIS goals and correct societal scientific illiteracy. The proceeds from these activities enabled us to award 2 scholarships to local high school teachers, enabling them to attend a workshop geared to updating their teaching skills and knowledge in a particular area. Commitments from industries / corporations will enable us in the future to award at least partial scholarships to outstanding female high school/college graduates interested in pursuing a scientific career.

Under the auspices of Detroit Area Pre-college Engineering Program (DAPCEP) together with Dr. M.A. Bednarski from Oakland University, I organized and taught a 10-week lecture/lab course in Genetics and Physiology to about 60 inner city pre-college students in Fall 1989. This course offering, on the campus of University of Detroit-Mercy, was the first "hands-on" laboratory experience for these students. In addition, my involvement in this activity served as a good advertisement for WSU, to which these students were exposed during my lectures and to which they may apply when choosing a

university. It is interesting to note that WSU recently established a middle school, a clear signal of part of the University's mission, especially an inner city such as WSU, to the community. My participation with DAPCEP clearly demonstrates part of that mission and service to the community.

On April 26, 1990, our AWIS Detroit area chapter, together with DAPCEP, sponsored 10 high school students to attend an informative breakfast briefing meeting of the Michigan Technology Council (Dr. R. Thomas, WSU director), Sheraton Southfield, Southfield, MI.

#### *Positions Held in Professional Associations*

President of AWIS, Detroit Chapter, June 1988 - June 1990.

#### **Professional Consultations**

Consultant to the Biochips Feasibility Study, supported by and submitted to the National Geno Sciences, Inc., 1982.

Enzo Biochem, Inc., 2000.

#### **Journal/Editorial Activities**

ad hoc reviewer for Gene (1980-present).

ad hoc reviewer for Proc. Natl. Acad. Sci. USA (1990-present).

reviewer for BioTechniques (1990-present).

reviewer for Insect Molecular Biology (1993-present).

#### **Other Professionally Related Service**

Participant in a) NSF, and b) MBRS site visits to WSU, c) visit by the Michigan legislators regarding the recombinant DNA technology/research going on in the Department of Biological Sciences.

Co-organizer of a Symposium at the AAAS meeting May 1983, Detroit, MI.

ad hoc reviewer for NSF, Genetic Biology Program, (1988-present).



ad hoc reviewer for NSF, Systematic Biology, (1989-present).

ad hoc reviewer for Harper & Row Publishing Company, Biology text, 1985, 1986.

ad hoc reviewer for Medical Research Council, London, England, 2000.

External reviewer of a proposal from the Department of Biochemistry, Ohio State University, requesting an endowed chair position for an eminent scholar in protein engineering. The program for the Eminent Scholar positions is sponsored and funded by the Board of Regents of the State of Ohio, 1985.

Member of review panels for predoctoral and postdoctoral programs by the National Research Council (NRC) which reviews such proposals for NRC, Ford Foundation and National Science Foundation Fellowship Programs, 1986-present.

Member on the American Awards Panel for the American Association of University Women, 1986-present.

Research Peer Review Committee member for American Heart Association, Michigan, 1988-1991.

Reviewer for Student Research Study Section, American Heart Association, Michigan, 1990, 1991.

Judge of student posters at the Second Annual Minority Research Programs Day, August, 1999.

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Gene mapping and gene enrichment by the avidin-biotin interaction: use of cytochrome-c as a polyamine bridge

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ABSTRACT

A modification of previously described methods of electron microscopic gene mapping and of gene enrichment based on the avidin-biotin interaction is presented. The modification consists of coupling cytochrome-c instead of pentane diamine to the oxidized 2', 3' terminus of an RNA by Schiff base formation and  $\text{BH}_4$  reduction. The RNA-cytochrome-c conjugate is purified by a simple chromatographic procedure; several biotins are attached to the cytochrome moiety by acylation. The extended arm between biotin and RNA gives efficient electron microscopic gene mapping of DNA:RNA-biotin hybrids with avidin-ferritin and avidin-polymethacrylate sphere labels and efficient gene enrichment by buoyant banding of DNA:RNA-biotin:avidin-spheres in  $\text{CsCl}$ . A 1400 fold enrichment (thus, 25% pure) and a 30% yield of long *Drosophila* DNA strands with 5S RNA genes is achieved.

INTRODUCTION

The preceding paper (1) describes a method of mapping, with a ferritin label, a short RNA:DNA hybrid region along a single stranded segment of DNA. The essential features of the method are: a) covalent attachment of biotin to the periodate oxidized 3' terminus of the RNA by a diamine bridge, using a simple diamine such as  $\text{NH}_2(\text{CH}_2)_3\text{NH}_2$ ; b) covalent attachment of avidin to the electron opaque label ferritin; c) hybridization of the covalent tRNA-biotin conjugate to a single stranded segment of DNA that contains the coding sequence (gene) for the tRNA; d) electron microscopic mapping of the position of the hybridized tRNA-biotin along the single strand segment of DNA after binding of ferritin-avidin to the biotin. This method gives a moderately satisfactory overall efficiency of gene labeling, and has been used to map the tRNA genes of HeLa mitochondrial DNA (2). We describe here an improved method in which we use a defined polyamine instead of a diamine as the bridge between the 3' end of the RNA and the carboxylic acid biotin. The polyamine used is the protein, cytochrome-c. This bridge is believed to have several advantages: a) it is probably more extended than the pentane diamine; b) several biotins can be attached to one RNA molecule; c) the

purification step for tRNA-biotin with the pentane diamine bridge involves elution from avidin sepharose at pH 2.5 in 6M guanidine hydrochloride. These fairly drastic conditions are replaced for purification of tRNA-cytochrome-c-biotin (tRNA-cc-biotin) by hydroxyapatite chromatography at neutral pH, thus diminishing the probability of chemical degradation of the labeled RNA. As a result, presumably, of items a, b, and/or c, the overall efficiency of gene labeling with ferritin in the electron microscope by the new method is an improvement over that achieved previously. d) with the new extended bridge but not with the diamine bridge, avidin attached to polymethylmethacrylate spheres (3) will label DNA:tRNA-biotin hybrids.

Furthermore, the binding of avidin-spheres by DNA:RNA-biotin molecules forms the basis for a gene enrichment procedure. The polymer spheres used have a density and molecular weight of about 1.25 g/ml and  $7.6 \times 10^7$  daltons, respectively. The spheres can be used as relatively massive floats to separate DNA:RNA-biotin-avidin-sphere molecules from unhybridized DNA strands by buoyant banding in CsCl, thus accomplishing gene enrichment. Manning, Pellegrini and collaborators have demonstrated this application of the avidin-biotin labeling approach for long RNA molecules, by enriching for the rDNA genes of *Drosophila* and for the histone genes of the sea urchin (4,5). In these cases, it is appropriate and convenient to attach cytochrome-c-biotin nonspecifically to the RNA by  $\text{CH}_2\text{O}$  crosslinks. In the present communication, we show that 3' terminal labeling of 5S RNA by cytochrome-c can be used for highly efficient gene enrichment of the *Drosophila* 5S RNA genes.

The basic reaction scheme of the present procedure is:

- 1) Oxidation of free 2', 3' OH ends of RNA to the dialdehyde with periodate.
- 2) Schiff base formation of the terminal dialdehyde with the polyamine, cytochrome-c, at relatively low ionic strength, and stabilization of the compound against dissociation and/or  $\beta$  elimination by  $\text{BH}_4^-$  reduction.
- 3) Purification of RNA-cytochrome-c from free RNA and free cytochrome-c by sequential chromatography on carboxymethyl cellulose (CMC) and on hydroxyapatite (HAP).
- 4) Covalent attachment of several biotin molecules to lysine  $\text{NH}_2$  groups of the cytochrome-c by acylation with the N-hydroxy succinimide (NHS) ester of the carboxylic acid biotin.
- 5) Hybridization of the RNA-cc-biotin to DNA.
- 6) Labeling with avidin-ferritin or avidin-spheres.

- 7) Gene mapping by electron microscopy or gene enrichment by banding in CsCl.

#### MATERIALS AND METHODS

Nucleic Acids. *E. coli* tRNA's,  $\phi 80$  and  $\phi 80$   $\text{psu}_3^-$  DNA's, were obtained as described (1). *Drosophila* (Dm) 4S and 5S RNA and the *Drosophila* plasmids, pCIT19 and pCIT12, were prepared as previously described (6,7). Unlabeled as well as  $^3\text{H}$ -labeled Dm DNA's were isolated from Schneider's line 2 tissue culture cells. Cells were labeled by the addition of 1.5 m Ci of  $^3\text{H}$ -thymidine (Amersham Radiochemical Center, 41 Ci/mmol) to 50 ml of cells grown in suspension with gentle swirling. Cell density at the first addition of label was  $1-2 \times 10^6$  ml; label was added in four equal fractions at 6 hr intervals, and cells grown for another generation (approximately 24 hrs) after the last addition. Cells were harvested by centrifugation at 2400 rpm at  $0^\circ\text{C}$  for 5 minutes, lysed by homogenization (10-15 strokes) in 0.5 M Tris base 0.025 M KCl, 5 mM  $\text{Mg}(\text{Ac})_2$ , 0.35 M sucrose, pH 7.6. DNA was prepared from this lysate by the procedure of Manning et al. (8). The specific activities of two separate preparations were  $5.4$  and  $0.84 \times 10^5$  cpm/ $\mu\text{g}$ .

Cytochrome-c. Commercial cytochrome-c (horse heart, type VI, Sigma) is contaminated with RNAase, which has approximately the same molecular weight and charge. RNAase was inactivated by treatment with iodoacetate by a modification of published procedures (9,10). Cytochrome-c (60 mg) was dissolved in 1 ml of 0.2 M NaAc buffer (pH 5.5). An equal weight of iodoacetate was added, the pH readjusted to 5.5 with concentrated NaOH, and the solution diluted to a final volume of 2 ml. The solution was incubated for 1 hr at  $55^\circ\text{C}$  and then dialyzed extensively at  $0^\circ\text{C}$  against 0.01 M sodium phosphate buffer (NaP), pH 6.8, and lastly against 0.1 M  $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$  buffer, pH 9.2.

Preparation and Purification of RNA-Cytochrome-c. tRNA or 5S RNA were heated at  $80^\circ$  for 1-8 min in 1 mM NaAc buffer pH 6.8, cooled, adjusted to 0.1 M NaAc buffer (pH 4.8) and treated with periodate as previously described (1). The amount of RNA used was 0.5 - 1 mg in 0.5 - 1 ml of reaction mixture.

Oxidized RNA was dialyzed against 0.1 M  $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$  buffer (pH 9.2) at  $0^\circ\text{C}$ . A 10-15 fold molar excess of cytochrome-c was added and the solution

#### ABBREVIATIONS

RNA-cc-biotin, 4S or 5S RNA-cytochrome-c-biotin; HAP, hydroxyapatite; CMC, carboxymethylcellulose; NHS-biotin, N-hydroxysuccinimide ester of biotin; NaP, sodium phosphate buffer (50:50 mono and dibasic sodium phosphate); Dm DNA or RNA, *Drosophila melanogaster* nucleic acids; EM, electron microscopy.

incubated for 1 hr at room temperature. A total of 2 mg  $\text{NaBH}_4$ /1 mg RNA was added in 4 portions over a period of 80 min. The solution was allowed to stand at room temperature for an additional 30 min and the  $\text{NaBH}_4$  decomposed by addition of 0.1 - 0.2 ml of 4 M NaAc buffer (pH 5.0). The contents were dialyzed at 0°C against 0.01 M NaP buffer, pH 6.8. All of the steps up to and including reduction with  $\text{NaBH}_4$  were performed in the dark.

For the spectrophotometric determination of concentration, we use molar extinction coefficients of  $9.64 \times 10^4$  at 410 m $\mu$ ,  $5.4 \times 10^5$  and  $8.3 \times 10^5$  at 260 m $\mu$  for cytochrome-c, 4S RNA, and 5S RNA, respectively.

Free cytochrome-c was removed from the reaction mixture by passage over a 3 x 1 cm column of carboxymethyl cellulose (CMC) that had been reequilibrated with 0.01 M NaP buffer. The CMC had been washed with acid and base according to the directions provided by the supplier. The sample was loaded and washed in 0.01 M NaP buffer. Free tRNA and the tRNA-cytochrome-c conjugate come through in the first wash.

Hydroxypatite (HAP, Bio-gel HTP from Bio-Rad) was hydrated by boiling for 10-20 min in 0.01 M NaP, pH 6.8, washed with 0.5 M NaP buffer, and re-equilibrated in 0.01 M NaP. For each washing the suspension was swirled gently and allowed to settle for 10-15 minutes before decanting the finer particles. The HAP was packed into a 2 x 0.5 cm column in 0.01 M NaP and the mixture of tRNA and tRNA-cytochrome from the CMC column applied. The column was successively washed with 10 ml volumes of 0.1 M, 0.15 M, 0.3 M and 0.5 M NaP buffer, pH 6.8. Fractions (0.5 - 1 ml) were collected, and assayed by spectrophotometry.

Addition of Biotin. The N-hydroxysuccinimidyl (NHS) ester of  $^{14}\text{C}$ -biotin was prepared as described (1). The 1:1 conjugate, tRNA-cytochrome-c, from the HAP column was treated with an approximately 100 fold excess of NHS- $^{14}\text{C}$ -biotin under conditions previously described (3). Free biotin was removed by dialysis against 0.01 M NaP and the tRNA-cc-biotin stored at -20°C.

EM Labels. Ferritin-avidin was a gift from L. Angerer (1). Polymethylmethacrylate spheres (a gift from N.D. Hershey) were conjugated to avidin as previously described (3). One of the sphere-avidin preparations was a gift of M. Pellegrini.

Heteroduplex formation and electron microscopy. Heteroduplex formation between Dm plasmids containing 4S or 5S genes with DNA of the vector, Colicin E1, has been described (6,7).

$\phi 80\text{h}/\phi 80 \text{ psu}_3$  heteroduplexes were formed as follows: a solution containing equal amounts of  $\phi 80\text{h}$  and  $\phi 80 \text{ psu}_3$  bacteriophage was treated with 20  $\mu\text{l}$

of 0.2 M EDTA (pH 8.0) for 30 min on ice. Complete lysis of the virions and denaturation of DNA was accomplished by addition of 20  $\mu$ l of 1 N NaOH for 10-15 min at room temperature. The mixture was neutralized with 30  $\mu$ l of 2.5 M Tris HCl. tRNA-cc-biotin was added and the volume was made to 200  $\mu$ l with 3X recrystallized formamide (99%, Matheson, Coleman and Bell). The final DNA and tRNA concentrations were 3  $\mu$ g/ml and 10-30  $\mu$ g/ml. Hybridization was performed by dialysis of this mixture against 40% 3X recrystallized formamide, 0.1 M Tris, 0.3 M NaCl, 1 mM EDTA, pH 8.0 at 40°C for 40-50 min. Subsequent manipulations were as described (2). The concentration of spheres-avidin in labeling experiments was approximately 100  $\mu$ g/ml. No removal of excess spheres-avidin was attempted, as an effective procedure to do so is not available.

All electron microscopy and measurements of molecular lengths were done as previously described (2, 6, 7). Single and/or double stranded  $\phi$ X 174 DNA (5370 nucleotides or nucleotide pairs, (11)), was used as a length standard.

Preparation of Dm  $^{125}$ I 5S RNA.  $^{125}$ I-5S RNA was prepared essentially according to Orosz and Wetmur (12). The reaction mixture contained, in the order of addition, the following: 10  $\mu$ l H<sub>2</sub>O (double distilled), 3-5  $\mu$ l Dm 5S rRNA (1.9 mg/ml in 0.01 M NaAc, pH 4.8), 3  $\mu$ l of 1 M NaAc (pH 5.0), 10  $\mu$ l  $^{125}$ I (Amersham, carrier free, 100  $\mu$ Ci/ml), and 3  $\mu$ l of freshly prepared TlCl<sub>3</sub> (ICN-K & K Laboratories, 18 mg/10 ml double distilled H<sub>2</sub>O). The mixture was incubated at 60° for 20 min in a sealed siliconized 50  $\mu$ l pipette. The contents were transferred to 1 ml of 0.1 M NaAc buffer (pH 5.0) containing about 50-60  $\mu$ g of Dm 18S and 28S rRNA, and dialyzed against 0.5 M NaCl, 0.015 M NaH<sub>2</sub>PO<sub>4</sub>,  $2 \times 10^{-4}$  M EDTA, pH 6.0 at 0° (2 x 500 ml) and at 60°C (2 x 500 ml) and at 0°C again until no counts were detected in the dialysate. The  $^{125}$ I-5S RNA preparation was then treated with 50  $\mu$ g/ml proteinase K (EM Laboratories, Inc.), phenol extracted, and further purified as described (4) or on Cs<sub>2</sub>SO<sub>4</sub> gradients. Specific activities obtained in the different preparations ranged from 0.2 -  $1 \times 10^8$  cpm/ $\mu$ g.

Solution Hybridization of  $^{125}$ I-5S RNA to DNA fractions. The contents of 5S genes in the several fractions of Dm DNA for the gene enrichment experiments were carried out by saturation hybridization using excess  $^{125}$ I-5S RNA in solution. DNA solutions were denatured in 0.2 M NaOH, neutralized, and adjusted to the 80% formamide hybridization solution described in the next section. All of the samples contained  $10^{-4}$  M KI in order to reduce background. Typical concentrations of DNA assayed in the respective fractions were 0.075 - 0.5  $\mu$ g/ml, 13-38  $\mu$ g/ml, and 26-400  $\mu$ g/ml, in the enriched, un-

fractionated, and depleted fractions, respectively. Reactions were carried out to a rot of 0.06 - 0.6 mol sec/liter. Samples were diluted 10 fold with 2 x SSC and treated at 37° for 1 hour with RNAase (100 µg/ml RNAase A, 4 units/ml T1 RNAase).

Gene Enrichment Procedure. RNA:DNA hybridization for the gene enrichment experiments was carried out in a high formamide solvent (13) which permits RNA:DNA hybridization but little or no DNA:DNA reassociation. Formamide was 3X recrystallized. DNA in 80% formamide, 2XSSC, was denatured by heating to 80°C for 10 min. A typical hybridization mixture contained 100 µg/ml Dm <sup>3</sup>H-DNA, 10 µg/ml Dm 5S RNA-cc-biotin, 150 - 1000 µg/ml Dm 18 + 28S rRNA, all in 2XSSC, 80% formamide at 45°C for 30 min (rot =  $6 \times 10^{-2}$  mol sec  $l^{-1}$  for the 5S RNA). The sample was dialyzed at 0°C against 0.1 M NaCl, 1 mM Tris, 1 mM EDTA, pH 8.5, and passed over a Sepharose 2B column (19 x 1 cm) to remove excess 5S RNA-cc-biotin. Elution volume has previously been calibrated with Dm <sup>3</sup>H-DNA and <sup>125</sup>I 5S RNA. The volume of the DNA fraction was reduced to about 100-500 µl by evaporation in a vacuum desiccator. In different experiments 50-150 µl of avidin-spheres (10-15 mg/ml) were added either during or after the evaporation. The solution was adjusted to 1 M NaCl and allowed to stand for 12-16 hours at room temperature or 48 hrs at 0°C. Spheres and DNA bound to spheres were separated from free DNA by banding in CsCl as described (4) except that centrifugation was performed for 48 hrs. The amounts of DNA in the different fractions were determined by <sup>3</sup>H counting. DNA was released from the spheres and the RNA hydrolyzed by treatment with 0.2 M NaOH at 100°C for 20 min (14) or at 37°C for 16 hours. The 5S gene content of the several fractions was determined as described above.

#### RESULTS AND DISCUSSION

Preparation and Purification of RNA-cytochrome-c-biotin. tRNA or 5S RNA was reacted with cytochrome-c as described. The first step in the purification of the reaction product from the starting materials is passage over a CMC column in 0.01 M NaP buffer. Spectrophotometric monitoring showed that neither tRNA nor tRNA-cytochrome-c binds to the column whereas the free cytochrome-c does. The crucial step in the purification of RNA-cytochrome-c from unreacted RNA is HAP chromatography. As shown in fig. 1, tRNA elutes from HAP in a 0.15 M NaP wash, whereas RNA-cytochrome-c is eluted by 0.3 M NaP. The absorbance peaks at 410 and 260 mµ show that the material being eluted at 0.3 M NaP is the conjugate with 1:1 molar ratio.

Several comments should be made about the procedure. By using a 10-15 fold excess of cytochrome-c to RNA we decrease the probability of forming